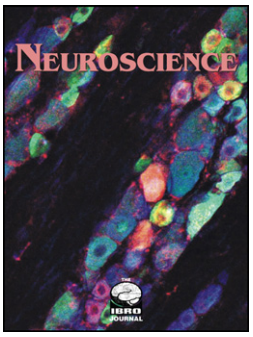
Accepted Manuscript



Neurotoxic effect of oligomeric and fibrillar species of A<beta>1-42 peptide. Involvement of ER calcium release in oligomers-induced cell death.

Rosa Resende, Elisabete Ferreiro, Claudia Pereira, Catarina Resende de Oliveira

PII: S0306-4522(08)00884-1
DOI: 10.1016/j.neuroscience.2008.06.036
Reference: NSC 10409

To appear in: *Neuroscience*

Received date: 15 November 2007
Revised date: 5 June 2008
Accepted date: 5 June 2008

Please cite this article as: Resende, R., Ferreiro, E., Pereira, C., Resende de Oliveira, C., Neurotoxic effect of oligomeric and fibrillar species of A<beta>1-42 peptide. Involvement of ER calcium release in oligomers-induced cell death., *Neuroscience* (2008), doi: 10.1016/j.neuroscience.2008.06.036.

Neurotoxic effect of oligomeric and fibrillar species of Aβ1-42 peptide. Involvement of ER calcium release in oligomers-induced cell death.

Rosa Resende, Elisabete Ferreiro, Cláudia Pereira, Catarina Resende de Oliveira

Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, 3004-504 Coimbra, Portugal

Corresponding author: Cláudia Pereira, Institute of Biochemistry, Faculty of Medicine and Center for Neuroscience and Cell Biology, University of Coimbra, 3004-504 Coimbra, Portugal

Tel: +351239820190; Fax: +351239822776; E-mail: cpereira@cnc.cj.uc.pt.

Article type: Research Paper

Section Editor: Dr. Constantino Sotelo, CNRS UMR 7102, Universite Pierre et Marie Curie, 6eme etage, Bat B, Case 12, 9 Quai St. Bernard, 75005 Paris, France

Abbreviations

Aβ, amyloid-beta peptide; AD, Alzheimer's disease; ADDLs, amyloid-derived diffusible ligands; APP, amyloid precursor protein; BSA, bovine serum albumine; CICR, Ca^{2+}-induced calcium release; DEVD-pNA, N-*acetyl*-Asp-Glu-Val-Asp-*p*-nitroanilide; DMSO, dimethyl sulfoxide; DT, dantrolene; DTT, 1,4-dithiotreitol; ECF, enhanced chemifluorescence; ER, endoplasmic reticulum; Fura-2/AM, Fura-2 acetoxymethyl ester; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; Indo-1/AM, indo-1 acetoxymethyl ester; IP_3R, inositol 1,4,5-triphosphate receptor; LTP, long-term potentiation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; MAP-2, microtubule-associated protein 2; NMDA, N-methyl-D-aspartate; PBS, phosphate-buffered saline; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RyR, ryanodine receptors; RT, room temperature; TBS, tris-buffered saline, ThS, thioflavin S; ThT, thioflavin T.

ABSTRACT

The nature of the toxic form of amyloid-β peptide (Aβ) early involved in Alzheimer's disease (AD) pathology and whether it is the fibrillar or the oligomeric peptide the most deleterious to neurons still remain controversial issues. This work was aimed to compare the neurotoxicity of different Aβ1-42 assemblies, using "fresh" and "aged" samples enriched in oligomeric and fibrillar species, respectively, and also isolated oligomers and fibrils. The results obtained with "fresh" and "aged" Aβ1-42 preparations suggested that oligomeric species are more toxic to cortical neurons in culture than fibrillar forms, which was confirmed by using isolated oligomers and fibrils. In order to further elucidate the mechanisms involved in soluble Aβ toxicity, the involvement of endoplasmic reticulum (ER) calcium release in oligomers-induced apoptosis was evaluated. We observed that oligomeric Aβ1-42 depletes ER Ca^{2+} levels leading to intracellular Ca^{2+} dyshomeostasis involving phospholipase C activation. Moreover, in the presence of dantrolene, an inhibitor of ER Ca^{2+} release through ryanodine receptors, the oligomers-induced apoptosis was prevented demonstrating the involvement of ER Ca^{2+} release.

KEYWORDS

Alzheimer's disease, amyloid-beta peptide, oligomers, fibrils, endoplasmic reticulum, calcium homeostasis.

The *in vitro* and *in vivo* neurotoxicity induced by fibrillar amyloid-beta peptide (Aβ) (Pike et al., 1993; Lorenzo and Yankner, 1994) has supported the amyloid cascade hypothesis in which seeding of insoluble Aβ1-42 is a causative factor in the pathogenesis of Alzheimer's disease (AD) (Hardy and Selkoe, 2002). However, amyloid plaques do not always correlate with neurodegeneration and cognitive decline (Masliah et al., 1994; Mucke et al., 2000; Klein et al., 2001). *In vivo*, small stable oligomers of Aβ1-42 have been isolated from the brain of AD patients (Gong et al., 2003) and the levels of soluble Aβ are well correlated with cognitive deficits (McLean et al., 1999; Naslund et al., 2000). Recent studies in animals have established a link between natural, as well as synthetic, soluble Aβ oligomers and cognitive impairment (Richardson et al., 2003; Cleary et al., 2005; Lesné et al., 2006). *In vitro*, oligomeric and protofibrillar forms of Aβ have been shown to be directly neurotoxic (Lambert et al., 1998; Hartley et al., 1999; Deshpande et al., 2006). Despite the significant advances in AD research made in the last decade, the nature of the toxic form of Aβ early involved in AD pathology and also whether it is the fibrillar or the non-fibrillar peptides that are the most deleterious to neurons still remain controversial issues. The above mentioned studies emphasize the necessity to clarify the initial response of neurons to the non-fibrillar and fibrillar Aβ, in particular to the Aβ1-42 peptide.

The endoplasmic reticulum (ER) has several important functions including the regulation of intracellular Ca^{2+} homeostasis. Under various conditions, ER function is disturbed leading to the accumulation of unfolded proteins and activation of a sporadic ER stress response, also known as the unfolded protein response (UPR). When cells are subjected to severe or prolonged ER stress, the transcriptional factor CHOP/Gadd153 is induced and apoptotic cell death occurs (Kaufman, 1999; Paschen, 2001). ER stress has been implicated in many important diseases, including AD (Lindholm et al., 2006). Ferreiro and colleagues (2006)

have recently demonstrated that ER Ca^{2+} release through ryanodine receptors (RyR) and inositol 1,4,5-triphosphate receptor (IP_3R) contributes to the early increase in intracellular Ca^{2+} levels and to the activation of apoptosis induced by fibrillar Aβ peptide. The present paper was aimed to compare the neurotoxic effects of different Aβ1-42 assemblies, namely oligomers and fibrils, using "fresh" and "aged" preparations of synthetic Aβ1-42 which are enriched in oligomeric or fibrillar species, respectively, and also isolated oligomers and fibrils. Furthermore, in this study we investigated the role of ER dysfunction in apoptosis induced by oligomeric Aβ1-42 in order to further elucidate the molecular mechanisms involved in Aβ oligomers-induced neurodegeneration. We have shown that oligomers are more toxic to cortical neurons than fibrils and induce apoptotic cell death through a phospholipase C- (PLC) mediated mechanism that involves ER Ca^{2+} release through channels associated with the RyR. Taken together, our findings support the hypothesis that oligomeric Aβ plays an important role in AD pathogenesis, being responsible for the early changes that lead to neuronal death.

EXPERIMENTAL PROCEDURES

Materials

Neurobasal medium and B27 supplement were purchased from GIBCO BRL, Life Technologies (Paisley, UK). Trypsin, trypsin inhibitor type-II-soybean, deoxyribonuclease I (DNase I), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), bovine serum albumine (BSA), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), U-73122 and Thioflavin T (ThT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetoxymethyl ester of Fura-2 (Fura-2/AM) and Indo-1 (Indo-1/AM), Hoechst 33342 and Alexa Fluor 594 goat anti-mouse IgG conjugate were purchased from Molecular Probes (Leiden, Netherlands). The synthetic Aβ1-42 peptide was from Bachem (Bubendorf, Switzerland). Phenol red-free Ham's F-12 medium was purchased from Cambrex Bio Science (Walkersville, USA). In Situ Cell Death Detection Kit, Fluorescein was obtained from Roche Applied Science (Mannheim, Germany). The caspase-3 colorimetric substrate N-*acetyl*-Asp-Glu-Val-Asp-*p*-nitroanilide (DEVD-pNA), ZVAD-fmk and MK801 were obtained from Calbiochem (Darmstadt, Germany). Mouse monoclonal antibody 6E10, reactive against residues 1-17 of Aβ was obtained from Signet (Deshman, MA, USA). Reagents and apparatus used in immunoblotting assays were obtained from Bio-Rad (Hercules, CA, USA), whereas polyvinylidene difluoride (PVDF) membrane, goat alkaline phosphatase-linked anti-mouse secondary antibody and the enhanced chemifluorescence (ECF) reagent were from Amersham Pharmacia Biotech (Buckinghamshire, UK). Low-Range Rainbow prestained protein standard was from Amersham Biosciences (USA). The Dako fluorescent mounting medium was purchased from DakoCytomation Inc. (Carpinteria, CA, USA). All the others chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck kgaA (Damstadt, Germany).

Primary rat embryo cortical neuronal cultures

Cortical neurons were isolated from E15-E16 Wistar rat embryos according to the method described by Hertz and collaborators (1989) slightly modified (Resende et al., 2007). Briefly, removed cortices were aseptically dissected and washed in Ca^{2+}- and Mg^{2+}-free Krebs buffer (in mmol/L): NaCl 120, KCl 4.8, KH_2PO_4 1.2, glucose 13, Hepes 10 (pH 7.4) and then incubated in Krebs solution supplemented with BSA (0.3 g/L), containing trypsin (0.5 g/L) and DNase I (0.04 g/L), for 10 min at 37 ºC. The tissue digestion was stopped by the addition of trypsin inhibitor (type II-S) (0.75 g/L) in Krebs buffer containing DNase I (0.04 g/L), followed by a centrifugation at 140 x *g* for 5 min. After washing the pellet once with Krebs buffer, the cells were dissociated mechanically and ressuspended in fresh Neurobasal medium with 2 mmol/L L-glutamine, 2% (v/v) B27 supplement, penincillin (100,000 U/L), and streptomycin (100 mg/L).

The cells were seeded on poly-L-lysine (0.1 g/L)-coated dishes at a density of 0.25 x 10^6 cells/cm^2 for measurement of caspase-3-like activity, 0.125 x 10^6 cells/cm^2 for the MTT assay or 0.33 x 10^6 cells/cm^2 for western blotting. For fluorescence studies, neurons were mounted on poly-L-lysine-coated glass coverslips at a density of 0.1 x 10^6 cells/ cm^2 or on poly-L-lysine-coated dishes at 0.4 x 10^6 cells/cm^2. The cultures were maintained in serum-free Neurobasal medium supplemented with B27, at 37 °C in a humidified atmosphere of 5% CO_2/95% air for 5-7 days before treatments in order to allow neuronal differentiation. Under these conditions, glial growth is less than 10% (Ferreiro et al., 2006).

Preparation of amyloid-β (Aβ) peptide solutions and treatment protocols

"Fresh and aged" peptide

The synthetic peptide Aβ1-42, corresponding to the neurotoxic amino acid residues of the human amyloid-beta protein (Aβ), was dissolved in sterile water, or in a diluted ammonia solution to facilitate peptide solubilization, at a concentration of 1 g/L (221.5 μmol/L). Aβ1-42 aliquots were then stored at -20 ºC until being used ("fresh" Aβ1-42 solution), or were incubated for 7 days at 37 ºC ("aged" Aβ1-42 solution).

The fibril content of both preparations ("fresh" and "aged" Aβ1-42) was analyzed by different criteria including the thioflavin T (ThT) fluorimetric assay and immunoblotting with the 6E10 antibody. To study the effect of the aggregation state of Aβ1-42 peptide on cell viability and apoptosis, different concentrations (0.5-5 μmol/L) of "fresh" or "aged" Aβ1-42 were added to the culture medium of cortical neuronal cells for 6-24 hr.

Aβ oligomers and fibrils

Synthetic Aβ1-42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to 1 mmol/L. The HFIP was then removed in a Speed Vac (Ílshin Lab. Co., Ltd, Ede, The Netherlands) and the dried HFIP film was stored at -20 ºC. The peptide film was resuspended to make a 5 mmol/L solution in anhydrous dimethyl sulfoxide (DMSO) (Dahlgren et al., 2002). Aβ1-42 oligomers were prepared by diluting the solution in phenol red-free Ham's F-12 medium without glutamine to a 100 μmol/L final concentration and incubated overnight at 4 ºC (Lambert et al., 1998). The preparation was centrifuged at 15,000 x *g* for 10 min at 4 ºC to remove insoluble aggregates, and the supernatant containing soluble oligomers was transferred to clean tubes and stored at 4 ºC. The fibrils were prepared by diluting 5 mmol/L Aβ1-42 in DMSO to 200 μmol/L in 100 mmol/L Hepes buffer (pH 7.5) and aged at 37 ºC for 7 days. The preparation was then centrifuged during 10 min at 15,000 x g at RT, and the supernatant containing soluble oligomers was discarded. The pellet containing Aβ fibrils (and possibly proto-fibrils) was ressuspended in 100 mmol/L Hepes buffer (pH 7.5). Protein

concentrations of Aβ oligomers and fibrils were determined using the Bio-Rad protein dye assay reagent.

Aggregation state of Aβ peptide

The fibril content in Aβ1-42 stock solutions was evaluated by a ThT fluorimetric assay as previously described (Hashioka et al., 2005) with some modifications. "Fresh" or "aged" Aβ1-42 peptide (221.5 μmol/L) was added to 3 μmol/L ThT in 50 mmol/L glycine-NaOH buffer (pH 8.5). Fluorescence was monitored at 450 nm excitation and 482 nm emission using a Perkin-Elmer LS50B spectrofluorometer. A time scan was performed and fluorescence values were measured after the decay reached a plateau and the background fluorescence of 3 μmol/L ThT was subtracted. The data from three identical samples in separate experiments were then averaged to obtain the final values.

The presence of different assembly forms (monomers, oligomers and fibrils) of Aβ1-42 in "fresh" and "aged" preparations and the purity of isolated oligomers and fibrils was evaluated by gel electrophoresis and western blot. Aβ samples containing 10 μg of protein, were diluted (1:2) with sample buffer [40% (w/v) glycerol, 2% (w/v) SDS, 0.2 mol/L Tris-HCl, pH 6.8 and 0.005% (w/v) Comassie G-250] and were separated by electrophoresis on a 4-16% Tris-Tricine SDS gel (Klafki et al., 1996). Samples were not boiled to minimize disaggregation prior to electrophoresis. To facilitate the identification of proteins a Low-Range Rainbow prestained protein standard was used. Proteins were then transferred to PVDF membranes, which were further blocked for 1 hr at RT with 5% (w/v) fat-free milk in Tris-buffered saline (150 nmol/L NaCl, 50 nmol/L Tris, pH 7.6) with 0.1% (w/v) Tween 20 (TBS-T). The membranes were next incubated overnight at 4 ºC with 6E10 mouse monoclonal primary antibody against Aβ diluted in TBS-T with 0.5% (w/v) fat-free milk (1:1000). After washing in TBS-T with 0.5% (w/v) fat-free milk, membranes were further incubated for 1 hr

at RT with an alkaline phosphatase-conjugated anti-mouse secondary antibody (1:20,000). Aβ bands were visualized after membrane incubation with ECF reagent for 5 min, on a Versadoc Imaging System.

Cell viability assay

After treatment of cortical neurons with different concentrations of the Aβ peptides, cell viability was evaluated by the MTT assay (Mosmann, 1983), which measures the ability of metabolic active cells to form formazan through cleavage of the tetrazolium ring of MTT. Neurons were washed in sodium medium (in mmol/L: NaCl 132, KCl 4, NaH_2PO_4 1.2, $MgCl_2$ 1.4, glucose 6, Hepes 10, and $CaCl_2$ 1, pH 7.4) and incubated with MTT (0.5 g/L) for 2 hr at 37 ºC. The blue formazan crystals formed were dissolved in an equal volume of HCl 0.04 mol/L in isopropanol and quantified spectrophotometrically by measuring the absorbance at 570 nm using a microplate reader (Spectra max Plus 384, Molecular Devices).

MAP2 immunocytochemistry

Cortical neurons grown in glass coverslips, in the presence or absence of Aβ1-42, were washed with PBS buffer (pH 7.4), and were fixed with 4% paraformaldehyde (w/v) for 15 min at RT. Then, the cells were permeabilized for 2 min at with 0.2% (v/v) Triton X-100 in PBS buffer (pH 7.4) and blocked for 1 hr and 30 min in PBS containing 3% (w/v) BSA. The cells were incubated for 1 hr with a mouse anti-microtubule-associated protein-2 (MAP-2) monoclonal antibody (1:500 dilution in 3% BSA/PBS) and then washed and incubated with Alexa Fluor 594 goat anti-mouse IgG antibody conjugate (1:200 dilution in 3% BSA/PBS) for 1 hr. Finally, coverslips were mounted in Dako fluorescent mounting medium on a microscope slide and neurons were visualized in an inverted fluorescence microscope Axiovert 200 (Zeiss, Germany).

Caspase-3 activity

Cultured cortical neurons, which were either treated or untreated with the Aβ peptide, were scrapped in cold (4 ºC) lysis buffer (in mmol/L): HEPES-Na 25, $MgCl_2$ 2, EDTA 1, EGTA 1, PMSF 0.1, DTT 2, supplemented with a protease inhibitor cocktail containing leupeptin, pepstatin A, chymostatin and antipain (1 g/L each). The cellular extract was rapidly frozen and thawed three times, and then centrifuged for 10 min at 20,200 x *g* at 4 ºC. The supernatant was collected and assayed for protein content using the Bio-Rad protein dye assay reagent. Aliquots of cellular extracts containing 30 µg of protein were reacted with 100 µmol/L Ac-DEVD-pNA, a chromogenic substrate for caspase-3, in a reaction buffer containing 25 mmol/L Hepes-Na, 10 mmol/L DTT, 10% (wt/vol) sucrose, and 0.1% (wt/vol) CHAPS (pH 7.4), for 2 hr at 37 ºC (Cregan et al., 1999). Caspase-3-like activity was determined by measuring substrate cleavage at 405 nm in a microplate reader.

Neuronal apoptosis

Apoptotic cells were identified and quantified based on nuclear DNA morphology by staining neurons with the cell-permeable DNA dye Hoechst 33342, as described previously by Kruman et al. (1997). Cortical neurons, incubated in the absence or in the presence of "fresh" Aβ, "aged" Aβ, oligomers or fibrils isolated from synthetic Aβ1−42, were washed with PBS buffer (pH 7.4) and fixed with 4% (wt/vol) paraformaldehyde for 15 min at RT. The cells were then incubated for 5 min with Hoechst 33342 (15 mg/L in PBS, pH 7.4), in the dark. Cells with homogeneously stained nuclei were considered to be viable, whereas the presence of condensed and fragmented nuclei was indicative of apoptotic cells. Cell death was also evaluated by TUNEL staining performed using an In Situ Cell Death Detection Kit, Fluorescein according to the manufacturer's directions. Fixed cells were permeabilized in

0.1% Triton X-100, supplemented with 0.1% sodium citrate in PBS, for 2 min, on ice. After washing, cells were incubated in a mixture of the enzymatic solution with the label solution, for 1 hr at 37 °C, in the dark. Finally, cells were washed with PBS and coverslips mounted in Dako fluorescent mounting medium on a microscope slide. DNAse pretreated cells were used as a positive control. The number of apoptotic cells was counted by using an inverted AxiovertMicroscope 200 (Zeiss, Germany).

Ca^{2+} homeostasis

The free intracellular cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$) was measured using Indo-1/AM. Untreated or treated cultured cortical neurons were incubated with 3 μmol/L Indo-1/AM in sodium medium (in mmol/L: NaCl 132, KCl 4, $CaCl_2$ 1, $MgCl_2$ 1.4, glucose 6, HEPES-Na 10, pH 7.4) for 45 min at 37 ºC, in the dark. The cells were then incubated in Indo-1/AM-free sodium medium for 15 min to ensure the complete hydrolysis of the dye. The Indo-1 fluorescence was measured spectrofluorimetrically using a microplater reader (SpectraMax Gemini EM, Molecular Devices) with 350 nm excitation and 410 nm emission. Calibration of cytosolic Ca^{2+} levels was performed using the Ca^{2+} ionophore ionomycin (3 μmol/L) and $MnCl_2$ (3 mmol/L). The free $[Ca^{2+}]i$ was calculated as previously described by Bandeira-Duarte et al. (1990).

Changes in cytosolic Ca^{2+} concentration in individual control or treated cells were analysed by calcium imaging using the fluorescent probe Fura-2/AM. Cells plated in coverslips were washed 2 times in Krebs buffer (pH 7.4) (in mmol/L: NaCl 132, KCl 4, $MgCl_2$ 1.4, Glucose 6, HEPES 10, $NaHCO_3$ 10, $CaCl_2$ 1) and loaded with 5 μmol/L Fura-2/AM supplemented with 0.2% (w/v) pluronic acid, in Krebs buffer for 40 min at 37 °C, in the dark. Afterwards, cells were washed 3 times and the coverslip was assembled to the perfusion chamber filled with 500 μL of Krebs buffer, in an inverted fluorescence microscope

axiovert 200 (Zeiss, Germany). Cells were alternately excited at 340 and 380 nm using a Lambda DG4 apparatus (Sutter Instruments company, Nocato, Ca, USA), and emitted fluorescence was collected with a 40x objective and was driven to a coll SNAP digital camera (Roper Scientific, Trenton, NJ, USA). After a baseline was established, cells were stimulated with Aβ oligomers (0.5 μmol/L). To assess extracellular Ca^{2+} contribution, recording of Fura2-AM fluorescence was also performed in Ca^{2+}- free Krebs buffer, supplemented with 50 μmol/L EGTA. To evaluate the involvement of PLC, cells were pre-incubated with PLC inhibitor U-73122 (5 μmol/L) for 10 min, before Fura2-AM fluorescence recording.

ER Ca^{2+} content was assessed by single cell Ca^{2+} imaging, according to the method described by Ferreiro and colleagues (2008). After a baseline was established in free Ca^{2+} -free Krebs buffer, cells were stimulated with thapsigargin (2.5 μmol/L final concentration), to empty $[Ca^{2+}]_{ER}$ stores. Acquired values were processed using the MetaFluor software (Universal Imaging Corporation, Buckinghamshire, UK). The peak amplitude of Fura-2 fluorescence (ratio at 340/380 nm) was used to evaluate cytosolic Ca^{2+} content.

Statistical analysis

Data were expressed as the means ± SEM of the indicated number of experiments. Statistical significance was determined by using one-way ANOVA followed by Tukey post-hoc tests. The differences were considered significant for p values < 0.05.

RESULTS

"Fresh" and "aged" Aβ1-42 peptide solutions have different fibril content

In this paper, we analyzed the effect of different Aβ1-42 assemblies on neuronal cell viability and apoptotic death. For that purpose, we prepared "fresh" and "aged" Aβ1-42 peptide solutions, which were characterized by ThT fluorescence and western blotting. The β-sheet content and, therefore, of fibrils of "aged" Aβ1-42, prepared after incubation of the peptide solution for 7 days at 37ºC, were significantly higher in comparison with that determined in "fresh" Aβ1-42, used immediately after peptide solubilization (Fig. 1A). Low-molecular weight oligomers of Aβ1-42 (~14 kDa) were preferentially detected in "fresh" samples while fibrillar Aβ1-42 (high molecular weight bands) was the predominant species detected in "aged" samples (Fig. 1B). Taken together, our results clearly show that "fresh" and "aged" Aβ1-42 preparations have a significantly different fibril content, the former being enriched in oligomeric forms and the later in fibrillar Aβ. Furthermore, the preformed fibrils of the "aged" peptide are not dissociated when the peptide is diluted into the culture medium as evaluated by fluorescence microscopy using thioflavin S (ThS) (data not shown). Thus, "fresh" and "aged" Aβ1-42 preparations are suitable to evaluate the differential effect of Aβ assemblies.

"Fresh" Aβ1-42 peptide is more toxic in comparison with the "aged" peptide

After 6 hr incubation of primary cortical neuronal cultures, in the absence or in the presence of increasing concentrations (0.5-5 μmol/L) of "fresh" or "aged" Aβ1-42 peptide, cell viability was analyzed by the MTT reduction assay. It was observed that at all tested concentrations, both "fresh" and "aged" Aβ1-42 are toxic to cortical neurons, significantly decreasing (p<0.001) the reduction of the tetrazolium salt (Fig. 2A). However, for the same

concentration (1 and 5 μmol/L), the "fresh" peptide was shown to be more toxic as compared to the "aged" peptide ($p<0.05$ and $p<0.001$).

Similar results were obtained when the number of apoptotic cells was determined in "fresh" or "aged" Aβ1-42-treated neurons (Fig. 2B). After 24 hr incubation, "fresh" Aβ1-42 was shown to be more effective than the "aged" peptide, significantly increasing the number of cells exhibiting apoptotic morphology as evaluated by using Hoechst 33342. These results were further confirmed using Tunel staining (Fig.4). Taken together, the results obtained demonstrate that Aβ1-42-induced toxicity and cell death correlate with the aggregation state of the peptide and suggest that the soluble oligomeric forms are the main toxins against cortical neurons.

"Fresh" Aβ1-42 peptide impairs Ca^{2+} homeostasis and leads to dendritic dystrophy and caspase-3 activation

As the "fresh" Aβ1-42 is more toxic than the "aged" peptide, we decided to perform some experiments using this peptide preparation enriched in oligomers in order to understand the mechanisms that underly the toxicity of soluble Aβ species. To investigate the effect of soluble Aβ on the intracellular Ca^{2+} homeostasis, the basal $[Ca^{2+}]i$ concentration was measured in control and in cortical neurons treated with "fresh" Aβ1-42 during 6 or 24 hr. Cytosolic Ca^{2+} levels were significantly higher ($p<0.05$) in cortical neurons treated with Aβ1-42 for 6 hr comparing with the control and this Ca^{2+} rise persisted 24 hr after addition of "fresh" Aβ1-42 (Fig. 3).

The analysis of dendritic morphology gives important information about neuronal health and synaptic integrity. We evaluated dendritic integrity in Aβ1-42-treated cortical neurons immunostained against MAP2 which was compared with that obtained in neurons treated with "aged" Aβ peptide (Fig. 4). Untreated cortical cells (control) showed a complex

dendritic network with smooth and long dendritic arborization (Fig. 4a). On the other hand, cultured neurons exposed to 5 μmol/L Aβ1-42 for 24 hr showed loss and retraction of the dendritic arborization (Fig. 4b, c). This effect is more pronounced in "fresh" Aβ-treated neurons (Fig. 4b).

The activity of caspase-3, an effector caspase in the apoptotic cell death pathway was measured in cortical neurons treated with 5 μmol/L "fresh" Aβ1-42 during 12 or 24 hr (Fig. 5A). Results obtained have demonstrated that "fresh" Aβ induces caspase-3 activation upon 12 hr treatment ($p<0.05$) which persists after 24 hr treatment. In order to evaluate the functional involvement of caspases in Aβ1-42-induced toxicity, ZVAD-fmk, a broad range caspase inhibitor was used. In the presence of 25 μmol/L ZVAD-fmk the increase in $[Ca^{2+}]i$ was prevented (Fig. 5B) suggesting the involvement of caspases in Aβ-induced Ca^{2+} dyshomeostasis. Furthermore, in the presence of ZVAD-fmk the increase in the number of apoptotic cells triggered by "fresh" Aβ1-42, determined by fluorescence microscopy using Hoechst 33342, was shown to be abolished (Fig. 5C).

Aβ1-42 oligomers are more toxic than fibrils

Our results obtained using "fresh" and "aged" Aβ1-42, enriched in oligomers and fibrils respectively suggest that soluble oligomeric forms of the Aβ peptide are more potent neurotoxins than fibrils. In order to validate these results, we isolated oligomers and fibrils accordingly to procedures previously published (Dahlgren et al., 2002; Lambert et al., 1998) which were characterized by immunoblotting with the 6E10 antibody. As shown in Fig. 6, oligomeric preparation (Aβ1-42O) is composed only of low-n oligomers (~14 kDa) and the fibrillar preparation (Aβ1-42f) is mainly composed of fibrils although smaller amounts of oligomers are also detected.

In cortical neurons, a significant decrease in cell viability was observed after 6 or 24 hr treatment with Aβ oligomers (p<0.01), as demonstrated by the MTT assay (Fig. 7A). At 0.5 and 1 μmol/L, Aβ1-42 oligomers were shown to be more toxic than fibrils (Fig 7B). At the same concentration, Aβ1-42 fibrils significantly decreased cell viability only after 24 hr treatment (p<0.05). Moreover, oligomers decreased cell survival more effectively than fibrils at all time points tested (p<0.05; p<0.001). The same results were obtained when the apoptotic cell number was determined after 24 hr treatment with oligomeric or fibrillar Aβ1-42 species (Fig. 7C). The increase in the number of apoptotic cells observed in oligomers-treated neurons was more pronounced (p<0.05) than that measured in cells incubated with fibrils. These data are in agreement with the results obtained with "fresh" and "aged" Aβ1-42 preparations.

Aβ oligomers-induced apoptosis is mediated through endoplasmic reticulum Ca^{2+} release

In cortical neurons treated with oligomeric Aβ1-42 (0.5 μmol/L) during 6 or 24 hr, the intracellular Ca^{2+} concentration was measured spectrofluorimetrically using Indo-1/AM. A significant increase in cytosolic Ca^{2+} levels was observed after 6 hr treatment with oligomeric Aβ compared with controls, which persisted until 24 hr incubation (Fig. 8). Dantrolene (DT), an inhibitor of ER Ca^{2+} release through channels associated to RyR, was shown to prevent the Aβ1-42 oligomers-induced rise of $[Ca^{2+}]_i$, suggesting the involvement of Ca^{2+} release by ER.

To further investigate the involvement of ER on the impairment of Ca^{2+} homeostasis triggered by Aβ oligomers, ER Ca^{2+} content was analyzed indirectly using thapsigargin, an inhibitor of the ER Ca^{2+} ATPase that releases Ca^{2+} from ER stores, in the absence of extracellular Ca^{2+}. A representative trace of Fura-2 fluorescence ratio at 340 nm and 380 nm is presented in Fig. 9A. After a baseline was established, cells were stimulated with thapsigargin, to empty $[Ca^{2+}]_{ER}$ stores. In control neurons, there was an increase in free

$[Ca^{2+}]i$ after the addition of thapsigargin. In treated neurons, this increase was not observed since Aβ oligomers have already induced the leak of Ca^{2+} from this intracellular organelle. The content of ER Ca^{2+} was evaluated by the calculation of the peak over baseline, obtained after thapsigargin addition and the results normalized to values determined in untreated cortical neurons. Figure 9B show that treatment with Aβ-42 oligomers for 1 hr induce a significant decrease in the ER Ca^{2+} content ($p<0.001$), which was more pronounced 24 hr after the addition of oligomers.

The inhibition of Ca^{2+} release from ER with DT (10 μmol/L) also prevented the apoptotic cell death that was observed in cortical neurons after exposure to Aβ1-42 oligomers (0.5 μmol/L) for 24 hr (Fig. 10). Together, these results demonstrate that the early release of Ca^{2+} from ER in cortical neurons exposed to oligomeric Aβ1-42 species leads to the perturbation of Ca^{2+} homeostasis and subsequently to apoptotic cell death.

IP3-PLC pathway is involved in the perturbation of ER Ca^{2+} homeostasis induced by Aβ oligomers

To analyze whether the entry of Ca^{2+} through Ca^{2+} channels present in the plasma membrane also contributes to the increase of cytosolic Ca^{2+} concentration upon treatment with Aβ oligomers, cortical neurons were loaded with the Ca^{2+}-sensitive fluorescent dye Fura-2/AM and the Ca^{2+} content in the cytosol was analyzed by single cell Ca^{2+} imaging in the presence or in the absence of extracellular Ca^{2+}. After 4 min recording, Aβ oligomers (0.5 μmol/L) were added to the cells. Few seconds after Aβ addition, Fura-2 fluorescence ratio at 340 nm and 380 nm (F340/F380) significantly increased in cells treated either in the presence or in the absence of Ca^{2+} in the medium. However, in the absence of Ca^{2+}, this increase was smaller than in the presence of Ca^{2+} (Fig. 11A). The increase in cytosolic Ca^{2+} content was determined by measuring the peak over baseline obtained after Aβ oligomers addition. Figure

11B depicts that the increase of cytosolic Ca^{2+} content is significantly higher in Aβ-treated cells in the presence of extracellular Ca^{2+} than in Ca^{2+}- free medium ($p<0.01$). These results indicate that the entry of Ca^{2+} through channels at the plasma membrane also contribute to the increase of intracellular Ca^{2+} induced by Aβ oligomers. To further investigate this hypothesis, we evaluated the involvement of NMDA receptors by using the MK801 which is an antagonist of these receptors. In the presence of 2 μmol/L MK801, the increase in the $[Ca^{2+}]i$ was partially prevented (Fig. 12A) but it does not affect the Aβ oligomers-induced apoptotic cell death (Fig. 12B) .

The increase in cytosolic Ca^{2+} content upon ER Ca^{2+} depletion induced by Aβ oligomers can be related to the activation of PLC, leading to the formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) that subsequently mediates the release of Ca^{2+} from ER through IP3R and potentiates Ca^{2+} release by RyR (Ca^{2+}-induced Ca^{2+} release, CICR) (Mikoshiba, 2006). To evaluate whether IP3-PLC pathway is involved in the $[Ca^{2+}]i$ increase induced by Aβ oligomers, cortical neurons were pre-incubated with the PLC inhibitor U-73122, loaded once again with Fura-2/AM, and stimulated with Aβ oligomers (0.5 μmol/L). The increase in F340/F380 ratio observed in Aβ oligomers-treated cells was prevented by U-73122 in the presence or absence of extracellular Ca^{2+}, suggesting the involvement of IP3-PLC signalling in the intracellular Ca^{2+} mobilization by Aβ oligomers (Fig. 11 A and B, $p<0.05$).

DISCUSSION

Aβ peptide exists in several different conformations, including monomers, oligomers, amyloid-derived diffusible ligands (ADDLs), protofibrils and fibrils. A strong correlation between soluble Aβ levels, and the extent of synaptic loss and cognitive impairment was described (Mclean et al., 1999; Klein et al., 2001; Lacor et al., 2004), suggesting that soluble oligomers of Aβ are the toxic assembly of the peptide that causes the synaptic dysfunction and dementia associated with the disease. However, the relative contribution of fibrillar and soluble oligomeric Aβ to neurodegeneration and dementia in AD has not yet been clearly established.

We here addressed the possibility that soluble oligomers and fibrillar (plaque-forming) Aβ1-42 peptide exert a differential effect on neurons. To test this hypothesis, we investigated the conformation-dependent effect on neuronal viability and activation of apoptotic cell death of different Aβ assemblies *in vitro*. Cortical neurons were exposed to "fresh" or "aged" Aβ1-42, which are enriched in low-molecular-weight oligomers (and possibly protofibrils) or have a significant high content in amyloid fibrils, respectively, as revealed by ThT staining and western blotting. By doing this, we intended to mimic what happens in the brain of AD patients in which the concentration of different Aβ1-42 assemblies varies depending on brain region, proximity to plaques and the stage of disease. The fact that the Aβ peptide exists *in vivo* as a mixture of peptides with different oligomerization states supports the use of our preparations ("fresh" and "aged" Aβ1-42) to directly compare the effects of distinct assemblies of Aβ. Our data, obtained using primary embryonic cortical neurons in culture treated with "fresh" or "aged" Aβ1-42, demonstrate that both assemblies of Aβ1-42 are toxic to neurons. However, in comparison with fibrils-enriched Aβ1-42 preparations, Aβ samples enriched in oligomers caused a significantly greater decrease in cell viability and a higher increase in the extent of apoptotic death than fibrils. In addition, an early activation of

apoptosis was shown to occur upon exposure to "fresh" Aβ1-42. Although the monomeric content is higher in "fresh" Aβ which was shown to be more toxic, it does not seem reasonable to attribute the toxicity to the presence of monomers. It has been shown that unlike the oligomers, the monomeric form of Aβ peptide does not increase intracellular Ca^{2+} levels (Demuro et al., 2005) and does not affect neither LTP (long term potentiation) (Walsh et al., 2002; Townsend et al., 2006) nor EPSC (excitatory post-synaptic current) activity (Cleary et al., 2005). Indeed, monomeric Aβ peptide has been described as antioxidant and neuroprotective (Zou et al., 2002). Therefore, the differential results obtained with "fresh" or "aged" Aβ1-42 can be attributed solely to the presence of soluble oligomers or fibrils, respectively. These results were validated using oligomers (low-n oligomers, probably trimers) and fibrils isolated from synthetic Aβ1-42 being the oligomers more toxic than fibrils. The fibrils preparation, obtained accordingly to previously published protocols, were shown to have not only fibrils but also oligomers. However, this preparation was not as toxic as the oligomeric Aβ preparation. We cannot exclude the hypothesis that the toxicity induced by the different samples of the Aβ peptide is due to the presence of the oligomers and if so, the fibrils can be protective. Indeed, Shimmyo and collegues (2007) recently demonstrated that structural changes in Aβ1-42 from a random coil to a beta-sheet-rich structure protect cortical neurons from apoptotic cell death and caspase-3 activation.

The neurotoxic effect of aggregated Aβ has been associated with oxidative stress (Behl et al., 1994; Pereira et al., 1999; Ferreiro et al., 2006; Resende et al., 2007), mitochondrial damage (Pereira et al., 1998; Cardoso et al., 2001; Casley et al., 2002) and intracellular Ca^{2+} dyshomeostasis (Mattson et al., 2002, Ferreiro et al., 2006). The fibrillar Aβ peptide has been shown to induce apoptosis in neurons involving both the mitochondrial-mediated and the ER stress-mediated apoptotic pathways (Ferreiro et al., 2004, 2006, *in press*) which may contribute to the neuronal degeneration in AD. In addition, neuritic

dystrophy and synaptic loss were shown to be induced by fibrillar Aβ (Grace et al., 2002; Grace and Busciglio, 2003; Resende et al., 2007). Deposition of fibrillar Aβ was also described to be associated with neurite breakage and permanent disruption of neuronal connections (Tsai et al., 2004). However, because insoluble protein aggregates are likely surrounded by oligomers it is difficult to ascertain whether the large aggregates are responsible for local neuronal dysfunction. Several *in vitro* studies as well as studies using animal models of AD show that soluble oligomeric forms (and protofibrils) of Aβ, intermediates in the formation of fibrils by synthetic Aβ, are the main neurotoxins for neurons which are responsible for the synaptic dysfunction that occurs in the early stages of the disease (Lambert et al., 1998; Hartley et al., 1999; Walsh et al., 2002). Naturally secreted low-n Aβ oligomers (dimers and trimers) induce progressive loss of hippocampal synapses (Shankar et al., 2007) and disrupt cognitive function (Cleary et al., 2005). Aβ oligomers disrupt Ca^{2+} homeostasis in primary neuronal cultures (De Felice et al, 2007b; Kelly et al., 2006). It has also been demonstrated that soluble oligomeric Aβ induces oxidative stress (De Felice et al., 2007) and tau phosphorylation (De Felice et al., 2007b; Resende et al., 2008). The oligomers that we obtained are low-n oligomers, probably trimers. Shankar and colleagues (2007) showed that naturally secreted Aβ dimers and trimers induce progressive loss of hippocampal synapses. Demuro and colleagues (2005) demonstrated the involvement of these forms in Ca^{2+} dyshomeostasis and proposed that a rise in intracellular Ca^{2+}concentration is mainly due to the oligomer-induced increase of the permeability of the plasma membrane. The present study demonstrate that "fresh" Aβ1-42, which is enriched in low molecular weight oligomeric forms (~14 kDa) perturbs Ca^{2+} homeostasis causing neuritic dystrophy and apoptotic cell death involving caspase-3 activation.

One major finding of this work is the involvement of ER Ca^{2+} release in oligomers-induced toxicity in cortical neurons. Here, we demonstrated that Aβ oligomers induce the early release of Ca^{2+} from ER, leading to the depletion of ER Ca^{2+} content after 1 hr treatment. In addition, using dantrolene, an inhibitor of ryanodine (RyR) receptors present in the ER, we also demonstrated that ER Ca^{2+} release is involved in the oligomeric Aβ-induced apoptotic cell death observed in cortical neurons. Furthermore, we show that Aβ oligomers also induce the increase of cytosolic Ca^{2+} through a process dependent of phospholipase C (PLC) signalling. The intracellular Ca^{2+} levels and the ER Ca^{2+} content is guaranteed by two different processes: the pumping of Ca^{2+} into the ER by SERCA ATPases and the release of Ca^{2+} by the opening of inositol 1,4,5-triphosphate receptor (IP_3R) or RyR Ca^{2+} releasing channels (Berridge et al., 2000), which participate in the signal transduction pathway of apoptosis (Guo et al, 1997; Jayaraman and Marks, 1997; Pan et al, 2000). The activation of IP_3R is indirectly linked to the activity of PLC. When G-protein coupled receptors are activated, phospholipase C causes the hydrolysis of phosphatidylinositol (4,5) biphosphate (PIP_2) to release IP_3 and diacylglycerol (DAG) (Dutta, 2000). IP_3 further reports the signal to the ER by activating IP_3R to release ER Ca^{2+}. Both IP_3R and RyR channels can be sensitized by cytosolic Ca^{2+}, resulting in a process called Ca^{2+}-induced Ca^{2+} release (Finch et al., 1991; Yao et al., 1992; Friel et al., 1992). CICR through RyR can also be triggered and amplified by Ca^{2+} released through IP_3R. It was previously shown that PLC is activated by Aβ25-35, the fragment that mimics full length Aβ peptide toxicity, and to be a Ca^{2+}-independent stimulation (Singh et al., 1997; Hedin et al., 2000) possibly due to a receptor-mediated event, activation of G-protein coupled receptors or direct interaction of Aβ with PLC. These data are in accordance with the results presented in this work, since the inhibitor of PLC was shown to have the same effect on the cytosolic Ca^{2+} increase induced by Aβ oligomers in the presence or in the absence of extracellular Ca^{2+}. Further studies are needed to elucidate how Aβ

oligomers activate PLC to release Ca^{2+} from the ER. Nevertheless, it appears that the production of IP_3 induced by Aβ oligomers leads to the release of Ca^{2+} through IP_3R that may induce CICR and activation of RyR, culminating in exaggerated intracellular Ca^{2+} levels.

The contribution of Ca^{2+} influx through channels at the plasma membrane to the increase in cytosolic Ca^{2+} content cannot be excluded since the rise in $[Ca^{2+}]i$ induced by Aβ is attenuated in Ca^{2+}-free medium. The excitoxicity induced by excessive Ca^{2+} influx through channels associated to glutamate NMDA receptors (NMDARs) can be one of the many mechanisms through which the Aβ peptide exerts its toxicity. Indeed, the involvement of NMDARs on perturbation of Ca^{2+} homeostasis triggered by Aβ peptides have been published (Domingues et al., 2007; Resende et al., 2007). In this work we also evaluated the involvement of NMDARs on Aβ oligomers-induced toxicity and results have shown that these Aβ species can induce Ca^{2+} influx through this subtype of glutamate receptors. However, blocking the NMDARs with the competitive antagonist MK801 did not protected from apoptotic cell death, suggesting that the extracellular Ca^{2+} has a relative contribution to the Aβ oligomers-induced toxicity.

The *in vitro* results presented here, obtained using "fresh" and "aged" Aβ1-42 preparations enriched in oligomers or fibrils, respectively, as well as isolated oligomeric and fibrillar species, are consistent with the view that soluble oligomeric forms of the Aβ peptide are the main neurotoxic species in AD that are able to initiate neuronal dysfunction in the early stages of the disease. Finally, we were able to demonstrate that the neurotoxicity induced by oligomeric forms of the Aβ peptide (Aβ1-42 isoform) involves the release of Ca^{2+} from the ER through channels associated with RyR. Oligomers can also activate IP3-PLC pathway leading to the release of Ca^{2+} through IP_3R that may induce CICR and activation of RyR, culminating in exaggerated intracellular Ca^{2+} levels.

ACKNOWLEDGEMENTS

The work was supported by the Research Support Division from the Faculty of Medicine, University of Coimbra, Portugal, and by the Portuguese Research Council (FCT). Rosa Resende and Elisabete Ferreiro are PhD fellows from FCT (SFRH/BD/11005/2002 and SFRH/BD/14108/2003, respectively).

REFERENCES

Bandeira-Duarte, C., Carvalho, C.A., Cragoe Júnior, E.J., Carvalho, A.P., 1990. Influence of isolation media on synaptosomal properties: intracellular pH, pCa, and Ca^{2+} uptake. Neurochem. Res. 15, 313-320.

Bahr, B.A., Hoffman, K.B., Yang, A.J., Hess, U.S., Glabe, C.G., Lynch, G., 1998. Amyloid β protein is internalized selectively by hippocampal field CA1 and causes neurons to accumulate amyloidogenic carboxyterminal fragments of the amyloid precursor protein. J. Comp. Neurol. 397, 139-147.

Behl, C., Davis, J.B., Lesley, R., Schubert, D., 1994. Hydrogen peroxide mediates amyloid beta protein toxicity. Cell 77, 817-827.

Billings, L.M., Oddo, S., Green, K.N., McGaugh, J.L., LaFerla, F.M., 2005. Intraneuronal abeta causes the onset of early Alzheimer's disease-related cognitive deficits in transgenic mice. Neuron 45, 675-688.

Berridge, M.J., Lipp, P., Bootman, M.D., 2000. Signal transduction. The calcium entry pas de deux. Science. 287, 1604-1605.

Cardoso, S.M., Santos, S., Swerdlow, R.H., Oliveira, C.R., 2001. Functional mitochondria are required for amyloid beta-mediated neurotoxicity. FASEB J. 15, 1439-1441.

Casley, C.S., Land, J.M., Sharpe, M.A., Clark, J.B., Duchen, M.R., Canevari, L., 2002. Beta-amyloid fragment 25-35 causes mitochondrial dysfunction in primary cortical neurons. Neurobiol. Dis. 10, 258-267.

Cleary, J.P., Walsh, D.M., Hofmeister, J.J., Shankar, G.M., Kuskowski, M.A., Selkoe, D.J., Ashe, K.H., 2005. Natural oligomers of the amyloid-beta protein specifically disrupt cognitive function. Nat. Neurosci. 8, 79-84.

Cregan, S.P., MacLaurin, J.G., Craig C.G., Robertson, G.S., Nicholson, D.W., Park, D.S., Slack, R.S., 1999. Bax-dependent caspase-3 activation is a key determinant in p-53-induced apoptosis in neurons. J. Neurosci. 19, 7860-7869.

Deshpande, A, Mina, E, Glabe, C, Busciglio, J., 2006. Different conformations of amyloid beta induce neurotoxicity by distinct mechanisms in human cortical neurons. J. Neurosci. 26, 6011-8.

Dahlgren, K.N., Manelli, A.M., Stine, W.B., Baker, L.K., Krafft, G.A., LaDu, M.J., 2002. Oligomeric and fibrillar species of amyloid-β peptides differentially affect neuronal viability. J. Biol. Chem. 277, 32046-32053.

De Felice, F.G., Velasco, P.T., Lambert, M.P., Viola, K., Fernandez, S.J., Ferreira, S.T., Klein, W.L., 2007. Abeta oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. J. Biol. Chem. 282, 11590-11601.

De Felice, F.G., Wu, D., Lambert, M.P., Fernandez, S.J., Velasco, P.T., Lacor, P.N., Bigio, E.H., Jerecic, J., Acton, P.J., Shughrue, P.J., Chen-Dodson, E., Kinney, G.G., Klein, W.L., 2007b. Alzheimer's disease-type neuronal tau hyperphosphorylation induced by Abeta oligomers. Neurobiol. Aging In press. doi:10.1016/j.neurobiolaging.2007.02.029.

Demuro, A., Mina, E., Kayed, R., Milton, S.C., Parker, I., Glabe, C.G., 2005. Calcium dysregulation and membrane disruption as a ubiquitous neurotoxic mechanism of soluble amyloid oligomers. J. Biol. Chem. 280, 17294-17300.

Dodart, J.C., Bales, K.R., Gannon, K.S., Greene, S.J., DeMattos, R.B., Mathis, C., DeLong, C.A., Wu, S., Wu, X., Holtzman, D. M., Paul, S.M., 2002. Immunization reverses memory deficits without reducing brain Aβ burden in Alzheimer's disease model. Nat. Neurosci. 5, 452-457.

Domingues, A., Almeida, S., da Cruz e Silva, E.F., Oliveira, C.R., Rego, A.C., 2007. Toxicity of beta-amyloid in HEK293 cells expressing NR1/NR2A or NR1/NR2B N-methyl-D-aspartate receptor subunits. Neurochem. Int. 50, 872-880.

Dutta, D., 2000. Mechanism of store-operated calcium entry. J. Biosci. 25, 397-404.

Ferreiro, E., Costa, R., Marques, S., Cardoso, S.M., Oliveira, C.R., Pereira, C.M., 2008. Involvement of mitochondria in endoplasmic reticulum stress-induced apoptotic cell death pathway triggered by the prion peptide PrP(106-126). J. Neurochem. 104, 766-776.

Ferreiro, E., Oliveira, C.R., Pereira C. The release of calcium from the Endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway. Neurobiol. Dis. *in press*

Ferreiro, E., Oliveira, C.R., Pereira, C., 2004. Involvement of endoplasmic reticulum Ca^{2+} release through ryanodine and inositol 1,4,5-triphosphate receptors in the neurotoxic effects induced by the amyloid-beta peptide. J. Neurosci. Res. 76, 872-880.

Ferreiro, E., Resende, R., Costa, R., Oliveira, C.R., Pereira, C.M., 2006. An endoplasmic-reticulum-specific apoptotic pathway is involved in prion and amyloid-beta peptides neurotoxicity. Neurobiol Dis. 23, 669-78.

Finch, E.A., Turner, T.J., Goldin, S.M., 1991. Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. Science. 252, 443-6.

Friel, D.D., Tsien, R.W., 1992. A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurones modulates effects of Ca^{2+} entry on $[Ca^{2+}]i$. J. Physiol. 450,217-246.

Gong, Y., Chang, L., Viola, K.L., Lacor, P.N., Lambert, M.P., Finch, C.E., Krafft, G.A., Klein, W.L., 2003. Alzheimer's disease-affected brain: presence of oligomeric Aβ ligands

(ADDLs) suggests a molecular basis for reversible memory loss. Proc. Natl. Acad. Sci. USA 100, 10417-10422.

Grace, E.A., Busciglio, J., 2003. Aberrant activation of focal adhesion proteins mediates fibrillar amyloid beta-induced neuronal dystrophy. J. Neurosci. 23, 493-502.

Grace, E.A, Rabiner, C.A, Busciglio, J., 2002. Characterization of neuronal dystrophy induced by fibrillar amyloid beta: implications for Alzheimer's disease. Neuroscience. 114, 265-273.

Guo, Q., Sopher, B.L., Furukawa, K., Pham, D.G., Robinson, N., Martin, G.M., Mattson, M.P., 1997. Alzheimer's presenilin mutation sensitizes neural cells to apoptosis induced by trophic factor withdrawal and amyloid beta-peptide: involvement of calcium and oxyradicals. J. Neurosci. 17, 4212-4222.

Gylys, K.H., Fein, J.A., Tan, A.M., Cole, G.M.., 2003. Apolipoprotein E enhances uptake of soluble but not aggregated amyloid-beta protein into synaptic terminals. J. Neurochem. 84, 1442-1451.

Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353-356.

Hartley, D.M., Walsh, D.M., Ye, C.P., Diehl, T., Vasquez, S., Vassilev, P.M., Teplow, D.B., Selkoe, D.J., 1999. Protofibrillar intermediates of amyloid β-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. J. Neurosci. 19, 8876-8884.

Hashioka, S., Monji, A., Ueda, T., Kanba, S., Nakanishi, H., 2005. Amyloid-β fibril formation is not necessarily required for microglial activation by the peptides. Neurochem. Int. 47, 369-376.

Hedin, H.L., Eriksson, S., Fowler, C.J., 2000. Human platelet calcium mobilisation in response to beta-amyloid (25-35): buffer dependency and unchanged response in Alzheimer's disease. Neurochem. Int. 38, 145-151.

Hertz, E., Yua, C.H., Hertz, L., Juurlink, B.H.J., Schousboe, A., 1989. Preparation of primary cultures of mouse cortical neurons. In *A dissection and tissue culture manual of the nervous system* (Shahar, A., De Vellis, J., Vernadakis, A., and Haber, B., eds), pp. 183-186, Alan R., Liss Inc., New York.

Jayaraman, T., Marks, A.R., 1997. T cells deficient in inositol 1,4,5-trisphosphate receptor are resistant to apoptosis. Mol. Cell Biol. 17, 3005-3012.

Kaufman, R.J., 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 13, 1211-1233.

Kim, H.J., Chae, S.C., Lee, D.W., Chromy, B., Lee, S.C., Park, Y.C., Klein, W.L., Krafft, G.A., Hong S.T., 2003. Selective neurodegeneration induced by soluble oligomeric amyloid beta-protein. FASEB J. 17, 118-120.

Klafki, H.-W., Wiltfang, J., Staufenbiel, M., 1996. Electrophoretic separation of βA4 peptides (1-40) and (1-42). Anal. Biochem. 237, 24-29.

Klein, W.L., Krafft, G.A., Finch, C.E., 2001. Targeting small Abeta oligomers: the solution to an Alzheimer's disease conundrum? Trends Neurosci. 24, 219-224.

Klyubin, I., Walsh, D.M., Lemere, C.A., Cullen, W.K., Shankar, G.M., Betts, V., Spooner, E.T., Jiang, L., Anwyl, R., Selkoe, D.J., Rowan, M.J., 2005. Amyloid β protein immunotherapy neutralizes Aβ oligomers that disrupt synaptic plasticity *in vivo*. Nat. Med. 11, 556-561.

Kriem, B., Sponne, I., Fifre, A., Malaplate-Armand, C., Lozac'h-Pillot, K., Koziel, V., Yen-Potin, F.T., Bihain, B., Oster, T., Olivier, J.-L., Pillot T., 2005. Cytosolic phospholipase

A2 mediates neuronal apoptosis induced by soluble oligomers of the amyloid-β peptide. FASEB J. 19, 85-7.

Kruman, I., Bruce-Keller, A.J., Bredesen, D.W., Waeg, G., Mattson, M.P., 1997. Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. J. Neurosci. 17, 5089-5100.

Lacor, P.N., Buniel, M.C., Chang, L., Fernandez, S.J., Gong, Y., Viola, K.L., Lambert, M.P., Velasco, P.T., Bigio, E.H., Finch, C.E., Krafft, G.A., Klein, W.L., 2004. Synaptic targeting by Alzheimer's related amyloid β oligomers. Neurobiol. Dis. 24, 10091-10200.

Lacor, P.N., Buniel, M.C., Furlow, P.W., Clemente, A.S., Velasco, P.T., Wood, M., Viola, K.L., Klein, W.L., 2007. Abeta oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease.J. Neurosci. 27, 796-807.

Lambert, M.P., Barlow, A.K., Chromy, B.A., Edwards, C., Freed, R., Liosatos, M., Morgan, T.E., Rozovsky, I., Trommer, B., Viola, K.L., Wals, P., Zhang, C., Finch, C.E., Krafft, G.A., Klein W.L., 1998. Diffusible, nonfibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins. Proc. Natl. Acad. Sci. USA 98, 6448-6453.

Lesné, S., Koh, M.T., Kotilinek, L., Kayed, R., Glabe, C.G., Yang, A., Gallagher, M., Ashe, K.H., 2006. A specific amyloid-β protein assembly in the brain impairs memory. Nature 440, 352-357.

Lindholm, D., Wootz, H., Korhonen, L., 2006. ER stress and neurodegenerative diseases. Cell Death Differ. 13, 385-392.

Lorenzo, A, Yankner, B., 1994. β-Amyloid neurotoxicity requires fibril formation and is inhibited by Congo red. Proc. Natl. Acad. Sci. USA 91, 12243-12247.

Manelli, A.M., Bulfinch, L.C., Sullivan, P.M., LaDu, M.J., 2007. Abeta42 neurotoxicity in primary co-cultures: effect of apoE isoform and Abeta conformation. Neurobiol. Aging. 28, 1139-47.

Masliah, E., Honer, W.G., Mallory, M., Voigt, M., Kushner, P., Hansen, L., Terry, R., 1994. Topographical distribution of synaptic-associated proteins in the neuritic plaques of Alzheimer's disease hippocampus. Acta Neuropathol (Berl). 87, 135-42.

Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I., Rydel, R.E., 2002. β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J. Neurosci. 12, 376-389.

Mclean C.A., Cherny R.A., Fraser F.W., Fuller S.J., Smith M.J., Beyreuther K., Bush A.I. and Masters, C.L., 1999. Soluble pool of Aβ amyloid as a determinant of severity of neurodegeneration in Alzheimer's disease. Ann. Neurol. 46, 860-866.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival. J. Immunol. Meth. 65, 55-63.

Mucke, L., Masliah, E,, Yu, G.Q., Mallory, M., Rockenstein, E.M., Tatsuno, G., Hu, K., Kholodenko, D., Johnson-Wood, K., McConlogue, L., 2000. High-level neuronal expression of Aβ 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. J. Neurosci. 20, 4050-4058.

Nagele, R.G., D'Andrea, M.R., Anderson, W.J., Wang, H.-Y., 2002. Intracellular accumulation of β-amyloid$_{1-42}$ in neurons is facilitated by the α7 nicotinic acetylcholine receptor in Alzheimer's disease. Neuroscience 110, 199-211.

Naslund, J., Haroutunian, V., Mohs, R., Davis, K.L., Davies, P., Greengard, P., Buxbaum, J.D., 2000. Correlation between elevated levels of amyloid beta-peptide in the brain and cognitive decline. JAMA 283, 1571-1577.

Nutt, L.K., Chandra, J., Pataer, A., Fang, B., Roth, J.A., Swisher, S.G., O'Neil, R.G., McConkey, D.J., 2002. Bax-mediated Ca^{2+} mobilization promotes cytochrome c release during apoptosis. J. Biol. Chem. 277, 20301-20308.

Pan, Z., Damron, D., Nieminen, A.L., Bhat, M.B., Ma, J., 2000. Depletion of intracellular Ca^{2+} by caffeine and ryanodine induces apoptosis of chinese hamster ovary cells transfected with ryanodine receptor. J. Biol. Chem. 275, 19978-19984.

Paschen, W., 2001. Dependence of vital cell function on endoplasmic reticulum calcium levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. Cell Calcium 29, 1-11.

Pereira, C., Santos, M.S., Oliveira, C., 1998. Mitochondrial function impairment induced by amyloid beta-peptide on PC12 cells. NeuroReport 9, 1749-1755.

Pereira, C., Santos, M.S., Oliveira, C., 1999. Involvement of oxidative stress on the impairment of energy metabolism induced by Abeta peptides on PC12 cells: protection by antioxidants. Neurobiol. Dis. 6, 209-219.

Pike, C.J., Burdick, D., Walencewicz, A.J., Glabe, C.G., Cotman, C.W., 1993. Neurodegeneration induced by β-amyloid peptides in vitro: the role of peptide assembly state. J. Neurosci. 13, 1676-1687.

Pillot, T., Drouet, B., Queillé, S., Labeur, C., Vandekerckhove, J., Rosseneu, M., Pinçon-Raymond, M., Chambaz, J., 1999. The non-fibrillar amyloid β-peptide induces apoptotic neuronal cell death: involvement of its C-terminal fusogenic domain. J. Neurochem. 73, 1626-1634.

Resende, R., Ferreiro, E., Pereira, C., Oliveira, C.R. ER stress is involved in Abeta-induced GSK-3beta activation and tau phosphorylation. J. Neurosci. Res. DOI: 10.1002/jnr.2164.

Resende, R., Pereira, C., Agostinho, P., Vieira, A.P., Malva, J.O., Oliveira, C.R., 2007. Susceptibility of hippocampal neurons to Abeta peptide toxicity is associated with perturbation of Ca^{2+} homeostasis. Brain Res. 1143, 11-21.

Richardson, J.C., Kendal, C.E., Anderson, R. et al., 2003. Ultrastructural and behavioural changes precede amyloid deposition in a transgenic model of Alzheimer's disease. Neuroscience 122, 213-228.

Roselli, F., Tirard, J.L., Hutzler, P., Lamberti, P., Livrea, P., Morabito, M., Almeida, O.F.X., 2005. Soluble β-amyloid$_{1-40}$ induces NMDA-dependent degradation of postsynaptic density -95 at glutamatergic synapses. J. Neurosci. 25, 11061-11070.

Saavedra, L., Mohamed, A., Ma, V., Kar, S., Posse de Chaves, E., 2007. Internalization of beta amyloid peptide by primary neurons in the absence of apoE. J Biol Chem. *In press.*

Shankar, G.M, Bloodgood, B.L, Townsend, M., Walsh, D.M., Selkoe, D.J., Sabatini, B.L., 2007. Natural oligomers of the Alzheimer amyloid-beta protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. J. Neurosci. 27:2866-2875.

Shimmyo, Y., Kihara, T., Akaike, A., Niidome, T., Sugimoto, H. 2007. Multifunction of myricetin on Abeta Neuroprotection via a conformational change of Abeta and reduction of Abeta via the interference of secretases. J Neurosci Res. *In press*.

Singh, I.N, Sorrentino, G., Kanfer, J.N., 1997. Amyloid beta protein (25-35) stimulation of phospholipase C in LA-N-2 cells. J Neurochem. 69, 252-258.

Sponne, I., Fifre, A., Drouet, B., Klein, C., Koziel, V., Pinçon-Raymond, M., Olivier, J.L., Chambaz, J., Pillot, T., 2003. Apoptotic neuronal cell death induced by the non-fibrillar amyloid-β peptide proceeds through an early ROS-dependent cytoskeleton perturbation. J. Biol. Chem. 278, 3437-3445.

Townsend, M., Shankar, G.M., Mehta, T., Walsh, D.M., Selkoe, D.J., 2006. Effects of secreted oligomers of amyloid beta-protein on hippocampal synaptic plasticity: a potent role for trimers. J. Physiol. 572, 477-492.

Tsai, J., Grutzendler, J., Duff, K., Gan, W.B., 2004. Fibrillar amyloid deposition leads to local synaptic abnormalities and breakage of neuronal branches. Nat. Neurosci. 11, 1181-1183.

Walsh, D.M., Klyubin, I., Fadeeva, J.V., Cullen, W.K., Anwyl, R., Wolfe, M.S., Rowan, M.J., Selkoe, D.J., 2002. Naturally secreted oligomers of amyloid β protein potently inhibit hippocampal long-term potentiation in vivo. Nature 416, 535-539.

Wang, H.W., Pasternak, J.F., Kuo, H., Ristic, H., Lambert, M.P., Chromy, B., Viola, K.L., Klein, W.L., Stine, W.B., Krafft, G.A., Trommer, B.L., 2002. Soluble oligomers of beta amyloid (1-42) inhibit long-term potentiation but not long-term depression in rat dentate gyrus. Brain Res. 924, 133-40.

White, J.A., Manelli, A.M., Holmberg, K.H., Van Eldik, L.J., LaDu, M.J., 2005. Differential effects of oligomeric and fibrillar amyloid-β1-42 on astrocyte-mediated inflammation. Neurobiol. Dis. 18, 459-465.

Yao, Y., Parker, I., 1992. Potentiation of inositol trisphosphate-induced Ca^{2+} mobilization in Xenopus oocytes by cytosolic Ca^{2+}. J. Physiol. 458,319-338.

Zou, K., Gong, J.S., Yanagisawa, K., Michikawa, M., 2002. A novel function of monomeric amyloid beta-protein serving as an antioxidant molecule against metal-induced oxidative damage. J. Neurosci. 22, 4833-4841.

LEGENDS

FIG. 1. Analysis of Aβ1-42 peptide fibrillogenesis in "fresh" and "aged" samples. (A) ThT fluorimetric assay in a cell-free system. Increased ThT fluorescence in "aged" peptide revealed greater β-sheet content in comparison with the "fresh" peptide. "Fresh" or "aged" (221.5 μmol/L) Aβ1-42 peptide was added to 3 μmol/L ThT in 50 mM glycine-NaOH buffer (pH 8.5) and fluorescence was monitored at 450 nm excitation and 482 nm emission. The data from three identical samples in separate experiments were then averaged to obtain the final values. *** $p<0.001$, significantly different from ThT fluorescence measured in "fresh" Aβ1-42 samples. (B) Western blotting. "Fresh" and "aged" Aβ1-42 samples were subjected to SDS-PAGE and the different assembly states of the Aβ peptide were detected, based on the molecular weight, using the monoclonal antibody (6E10) against Aβ. The analysis of the representative gel from three independent experiments shows that "fresh" and "aged" Aβ1-42 samples are enriched in oligomers or fibrils, respectively.

FIG. 2. "Fresh" Aβ1-42 peptide is more toxic to cortical neurons than "aged" peptide. A) Cultured cortical neurons were treated for 6 hr with increasing concentrations (0.5-5 μmol/L) of synthetic Aβ1-42 in a soluble or fibrillar assembly form (peptide added from "fresh" or "aged" solution, respectively). After that, cell viability was evaluated by measuring the capacity of the cells to reduce MTT. The results were presented as the percentage of the absorbance determined for control conditions and represent the means ± SEM of at least three independent experiments performed in duplicate. B) Primary cortical neurons cultured in glass coverslips were incubated for 24 hr with 1 or 5 μmol/L of both forms ("fresh" or "aged") of the synthetic Aβ1-42 peptide. Thereafter, apoptotic cells were quantified by nuclei morphology using Hoechst 33342. Values are means ± SEM of at least three independent

experiments performed in duplicate and correspond to the number of apoptotic cells as a percentage of total cells analyzed per sample. ***p<0.001, significantly different from control, in the absence of Aβ treatment. #p<0.05, ##p<0.01 significantly different from cortical cultures exposed to the same concentration of "aged" Aβ peptide.

FIG. 3. Basal $[Ca^{2+}]_i$ is increased upon treatment with "fresh" Aβ1-42. Aβ peptide increases basal intracellular Ca^{2+} levels. Primary cortical neurons treated with 5 μmol/L Aβ1-42 for 6 or 24 hr and the controls were incubated with Indo-1/AM for 45 min. After a 15 min post-loading period, the fluorescence of this Ca^{2+} indicator was measured and the $[Ca^{2+}]$i was calculated. *P<0.05, significantly different from control, in the absence of Aβ treatment.

FIG. 4. Dendritic network dystrophy induced by Aβ1-42. Control neurons exhibit long and smooth dendrites, while in Aβ-treated neurons dendritic loss and retraction and the presence of dystrophic dendrites is evident with "fresh" Aβ-treated neurons showing a more pronounced effect. Primary neurons cultured in glass coverslips (control or treated with 5 μmol/L "fresh" or "aged" Aβ1–42 for 24 hr) were fixed and immunostained with a mouse anti-MAP2 monoclonal antibody (red). Apoptotic cells are stained with Tunel (green). Representative images of control and of Aβ1-42-treated neurons were obtained from three independent experiments. 630 X.

FIG. 5. "Fresh" Aβ1-42 peptide induces caspase-3 activation. A) After treatment with 5 μmol/L Aβ1-42 ("fresh" form) for 12 or 24 hr, cultured cortical neurons were lysed and the cytosolic proteins were extracted. Caspase-3-like activity was determined by measuring cleavage of the chromogenic substrate Ac-DEVD-pNA at 405 nm and was expressed as arbitrary units of absorbance and the results represent the means ± SEM of at least three

independent experiments. B) Basal intracellular Ca^{2+} levels were measured in primary cortical neurons using the fluorescent Ca^{2+} indicator Indo-1/AM. Controls and neurons treated with 5 μmol/L "fresh"Aβ for 6 hr, in the absence or in the presence of 25 μmol/L ZVAD-fmk were incubated with Indo-1/AM for 45 min, followed by a 15 min post-loading period. Finally, Indo-1 fluorescence was recorded and the cytosolic Ca^{2+} concentration was calculated upon calibration with ionomycin and $MnCl_2$. C) Primary cortical neurons cultured in glass coverslips were incubated for 24 hr with 5 μmol/L of "fresh" Aβ1-42 peptide in the presence or in the absence of 25 μmol/L ZVAD-fmk. Thereafter, apoptotic cells were quantified by nuclei morphology using Hoechst 33342. Values are means ± SEM of at least three independent experiments performed in duplicate and correspond to the number of apoptotic cells as a percentage of total cells analyzed per sample. *P<0.05; ****p*<0.001, significantly different from control, in the absence of Aβ treatment. ##*p*<0.01; ###*p*<0.001, significantly different from cortical cultures exposed to 25 μmol/L ZVAD-fmk.

FIG. 6. Aggregation state analysis of isolated Aβ1-42 oligomers and fibrils. Fibrils and oligomers isolated from synthetic Aβ1-42 were subjected to SDS-PAGE. Proteins were immunoblotted with an anti-Aβ monoclonal antibody (6E10) and immunopositive bands were visualized by chemifluorescence. The gel depicts one of three independent experiments that gave similar results.

FIG. 7. Aβ1-42 oligomers are more toxic to cortical neurons than Aβ fibrils. A-B) Cultured cortical neurons were treated for 6 or 24 hr with oligomers (AβO) or fibrils (Aβf) isolated from synthetic Aβ1-42 (0.5 or 1 μmol/L). After that, cell viability was evaluated by measuring the ability of the cells to reduce MTT. The results were presented as the percentage of the absorbance determined for control conditions (no treatment with Aβ peptide) and

represent the means ± SEM of at least three independent experiments performed in duplicate. C) Primary cortical neurons cultured in glass coverslips were incubated for 24 hr with oligomeric (AβO) or fibrillar (Aβf) Aβ1-42 (0.5 μmol/L). Thereafter, apoptotic cells were quantified by nuclei morphology analysis using Hoechst 33342. Values are means ± SEM of at least three independent experiments performed in duplicate and correspond to the number of apoptotic cells as a percentage of total cells analyzed per sample. *p<0.05; **p<0.01; ***p<0.001, significantly different from control, in the absence of Aβ treatment. #p<0.05, ##p<0.01; ###p<0.001 significantly different from cortical cultures exposed to the same concentration of AβO or Aβf.

FIG. 8. Dantrolene prevents the Aβ1-42 oligomers-induced increase on basal $[Ca^{2+}]_i$. Basal intracellular Ca^{2+} levels were measured using the fluorescent Ca^{2+} indicator Indo-1/AM in primary cortical neurons. Controls and neurons treated with 0.5 μmol/L Aβ oligomers (AβO) for 6 or 24 hr, in the absence or in the presence of 10 μmol/L dantrolene (DT) were incubated with Indo-1/AM for 45 min, followed by a 15 min post-loading period. Finally, Indo-1 fluorescence was recorded and $[Ca^{2+}]$i was calculated upon calibration with ionomycin and $MnCl_2$. *P<0.05; **P<0.01, significantly different from control, in the absence of Aβ treatment. #p<0.05, significantly different from cortical cultures exposed to 10 μmol/L dantrolene.

FIG. 9. Depletion of ER Ca^{2+} content is induced by Aβ1-42 oligomers. Controls and cortical neurons treated with 0.5 μmol/L oligomers (AβO) for 1 or 24 hr were loaded with 5 μmol/L Fura-2/AM for 40 min at 37 °C, in the absence of extracellular Ca^{2+}, and changes in fluorescence ratio (F340/F380) were monitored during 2 min. Thapsigargin (2.5 μmol/L) was then added to empty ER Ca^{2+} stores and fluorescence ratio was monitored for an additional 10

min period. A) Representative trace of Fura-2 fluorescence ratio is presented. B) The peak amplitude of Fura-2 fluorescence was used to evaluate ER Ca^{2+} content. Data from at least three independent experiments were expressed as the decrease above control values. Data were expressed as the means ± SEM. ***p<0.001, significantly different from control.

FIG. 10. Apoptosis induced by Aβ1-42 oligomeric species is prevented by dantrolene. Cultured cortical neurons were treated for 24 hr with 0.5 μmol/L of Aβ1-42 oligomers (AβO) in the presence or in the absence of 10 μmol/L dantrolene (DT). The number of apoptotic cells was quantified in untreated and treated cortical neurons by fluorescence microscopy using Hoechst 33342. The results were presented as the percentage of the total number of cells and represent the means ± SEM of at least three independent experiments performed in duplicate. ****p*<0.001, significantly different from control, in the absence of AβO treatment. ###*p*<0.001 significantly different from cortical cultures exposed to AβO alone.

FIG. 11. Plasma membrane Ca^{2+} channels and phospholipase C activation contributes to Aβ oligomers-induced increase in $[Ca^{2+}]_i$. Cortical neurons were loaded with 5 μmol/L Fura-2/AM for 40 min at 37 °C, either in the absence (-Ca^{2+}) or in the presence (+Ca^{2+}) of extracellular Ca^{2+}, and changes in fluorescence ratio (F340/F380) were monitored during 4 min. Aβ oligomers (AβO, 0.5 μmol/L) were added to the cells and fluorescence ratio was monitored for an additional 8 min period. A group of cells were also pre-incubated with PLC inhibitor U-73122 (5 μmol/L) for 10 min, before Fura2-AM fluorescence recording. A) Representative trace of Fura-2 fluorescence ratio is presented. B) The peak amplitude of Fura-2 fluorescence was used to evaluate cytosolic Ca^{2+} content. Data from at least three independent experiments were expressed as the increase above control values. Data were expressed as the means ± SEM. **p<0.01, significantly different from control. # p<0.05

significantly different from AβO-treated cells. § $p<0.05$ significantly different from AβO-treated cells in the presence of extracellular Ca^{2+}.

FIG. 12. MK801 partially prevents increase on basal $[Ca^{2+}]_i$ but does not prevent apoptosis induced by Aβ1-42 oligomeric species. Cultured cortical neurons were treated for 6 or 24 hr with 0.5 μmol/L of Aβ1-42 oligomers (AβO) in the presence or in the absence of 25 μmol/L ZVAD-fmk. A) Basal intracellular Ca^{2+} levels were measured using fluorescent Ca^{2+} indicator Indo-1/AM. Primary cortical neurons were incubated with Indo-1/AM for 45 min, followed by a 15 min post-loading period. Finally, Indo-1 fluorescence was recorded and the cytosolic Ca^{2+} concentration was calculated upon calibration with ionomycin and $MnCl_2$. B) The number of apoptotic cells was quantified in untreated and treated cortical neurons by fluorescence microscopy using Hoechst 33342. The results were presented as the percentage of the total number of cells and represent the means ± SEM of at least three independent experiments performed in duplicate. $^{**}p<0.01$ $^{***}p<0.001$, significantly different from control, in the absence of AβO treatment.

Figure
Click here to download high resolution image

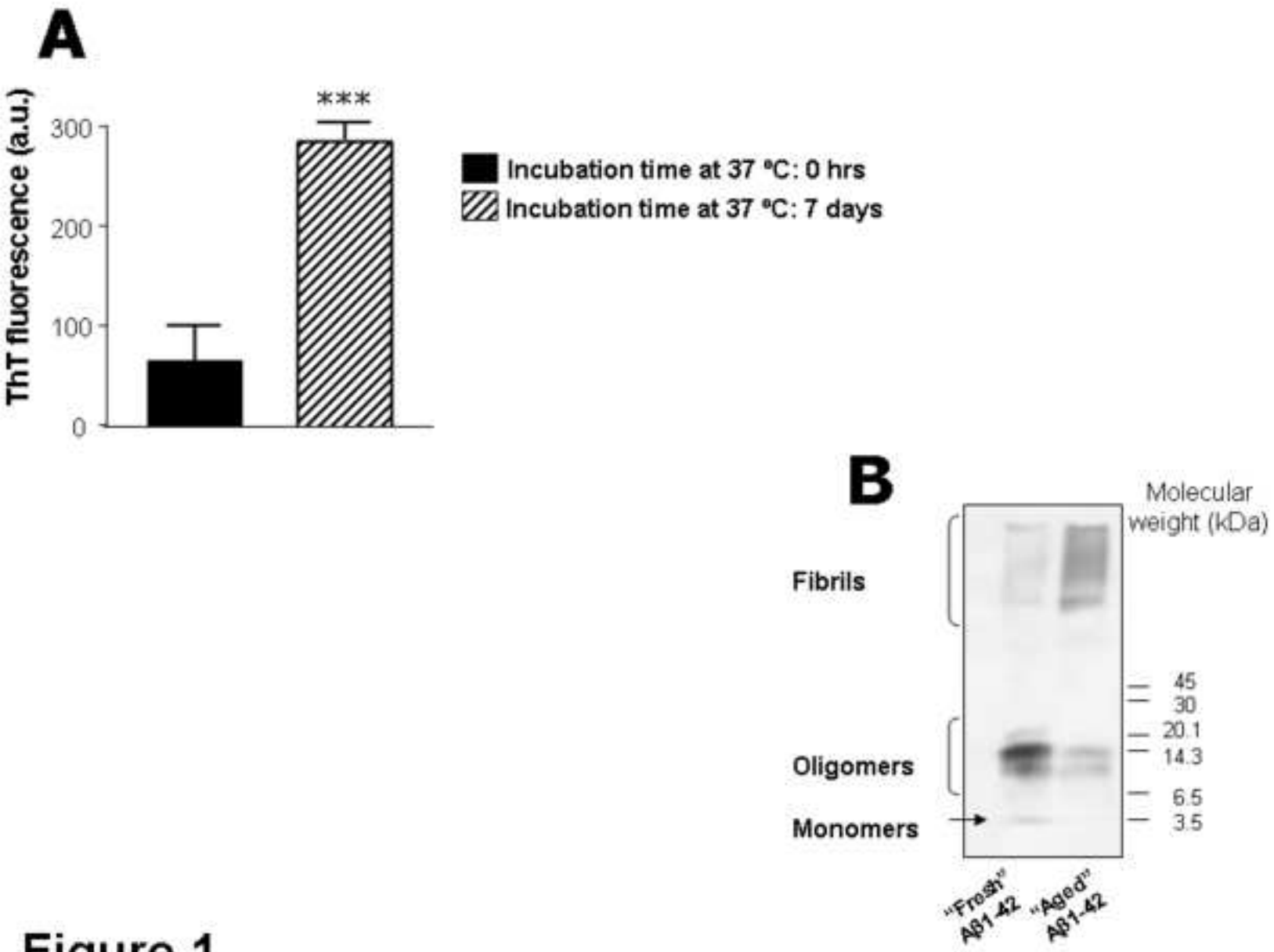


Figure 1

Figure
Click here to download high resolution image

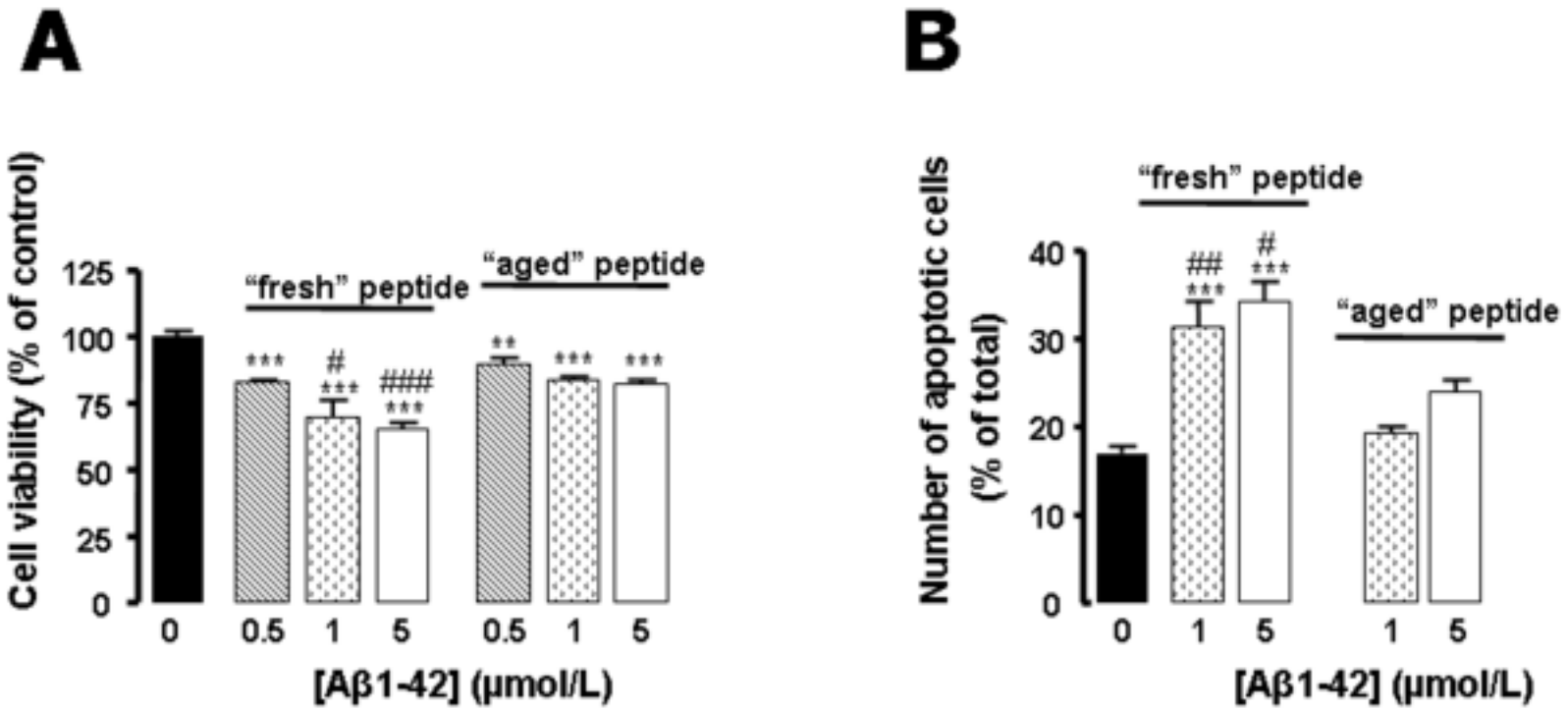


Figure 2

Figure
Click here to download high resolution image

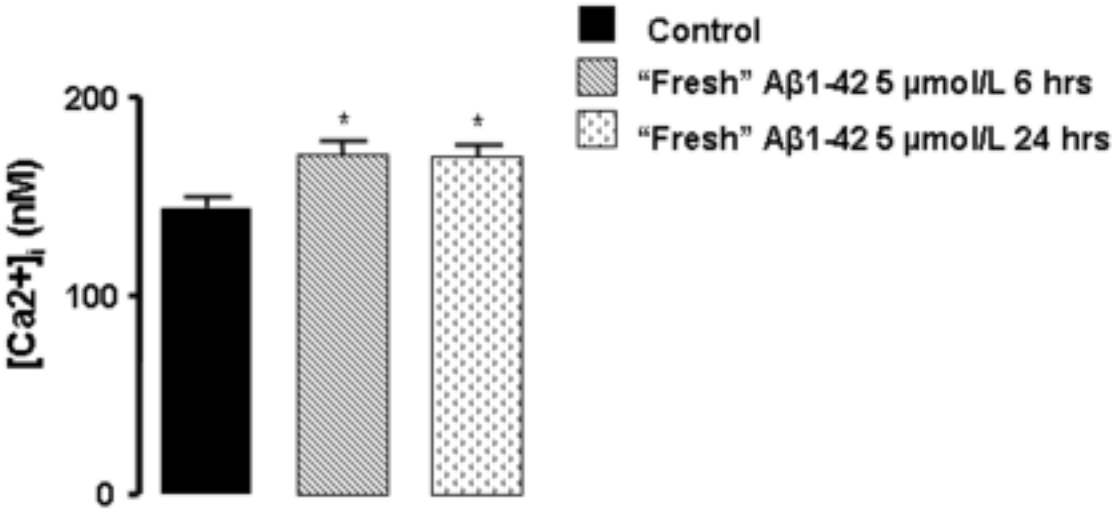


Figure 3

Figure
Click here to download high resolution image

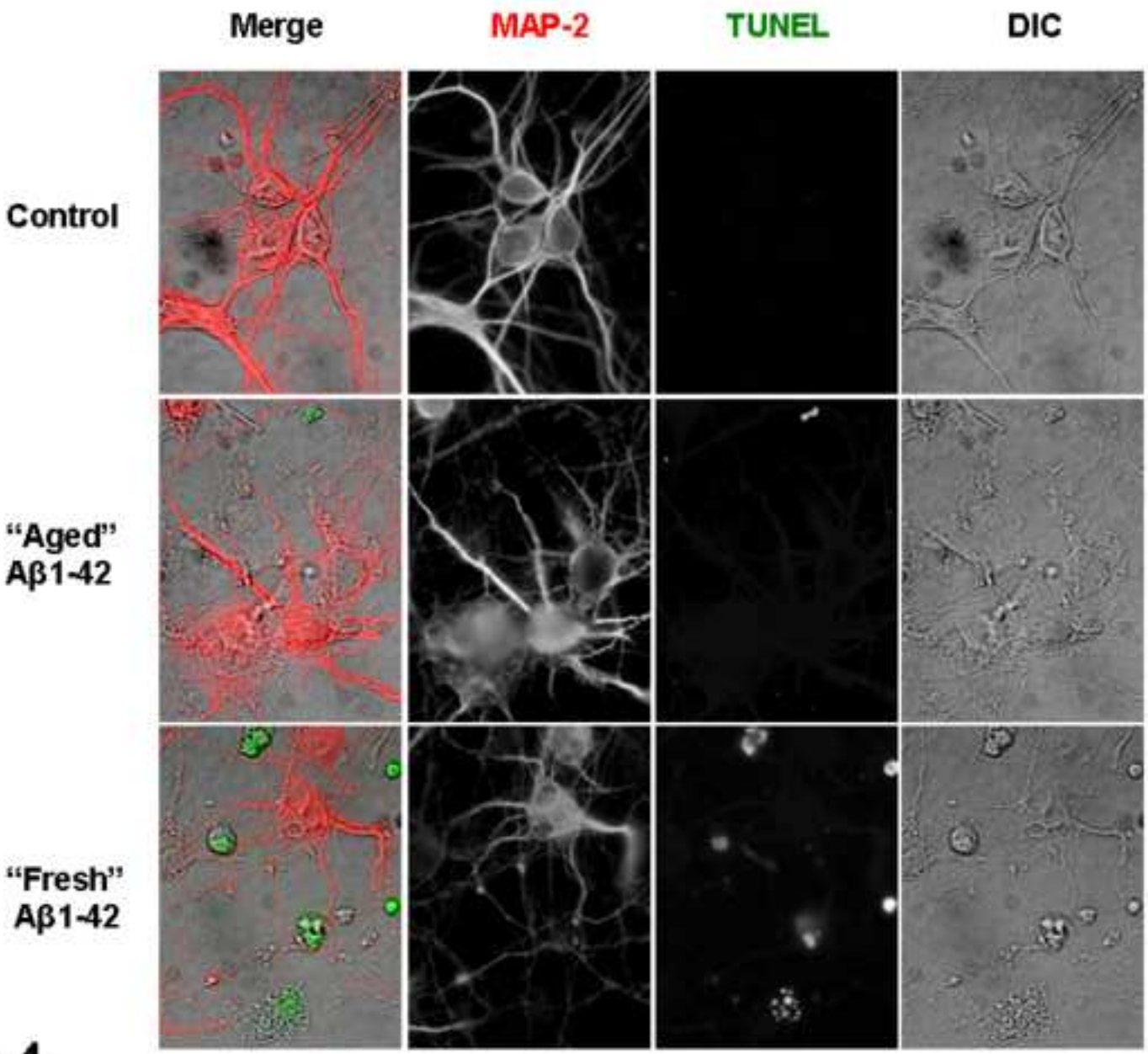


Figure 4

Figure
Click here to download high resolution image

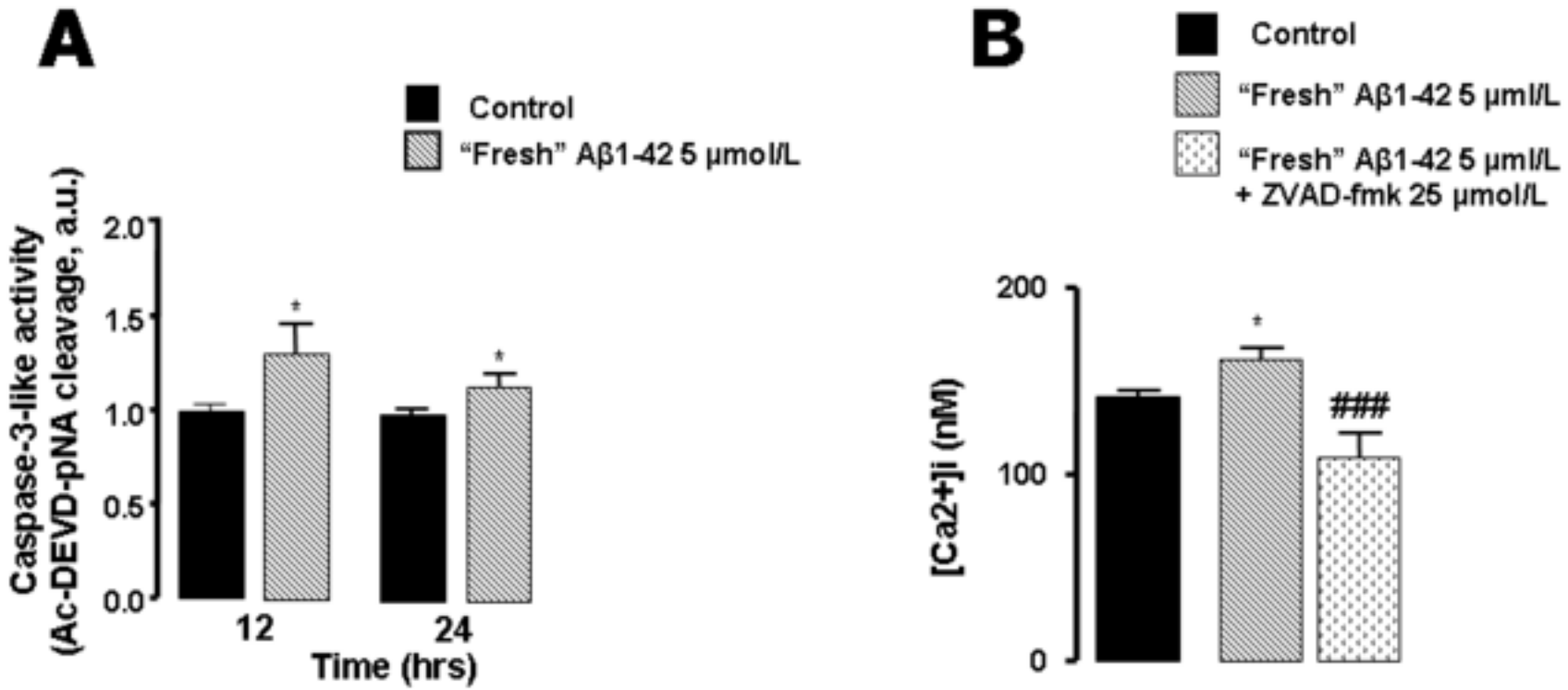


Figure 5

Figure
Click here to download high resolution image

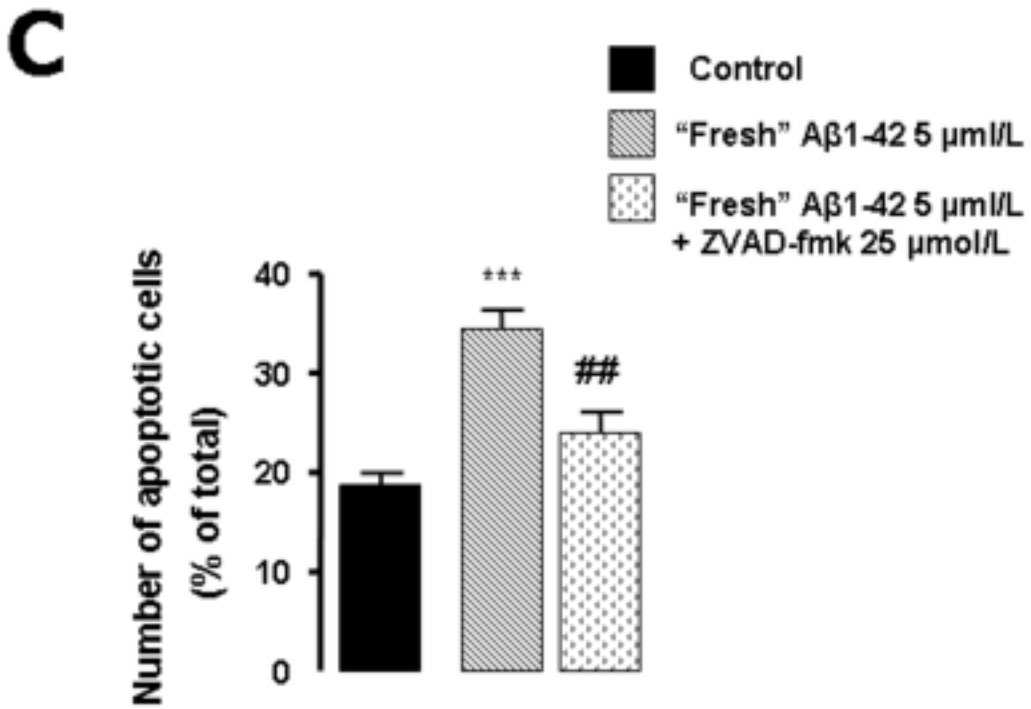


Figure 5

Figure
Click here to download high resolution image

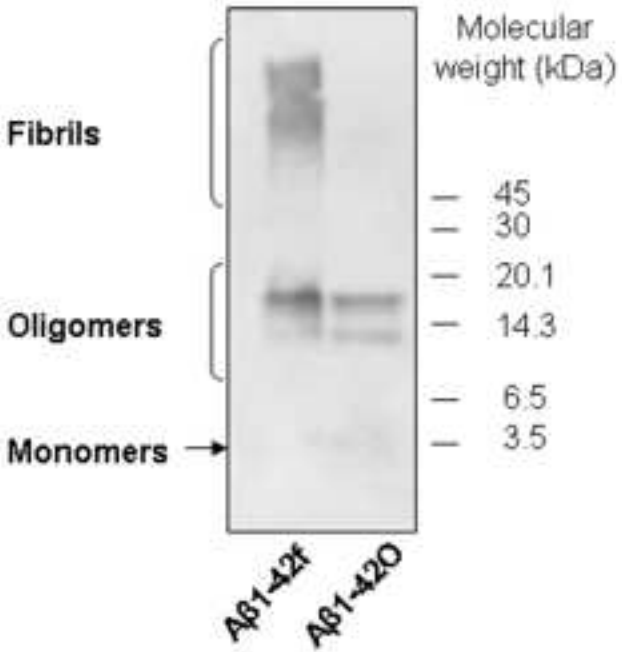


Figure 6

Figure
Click here to download high resolution image

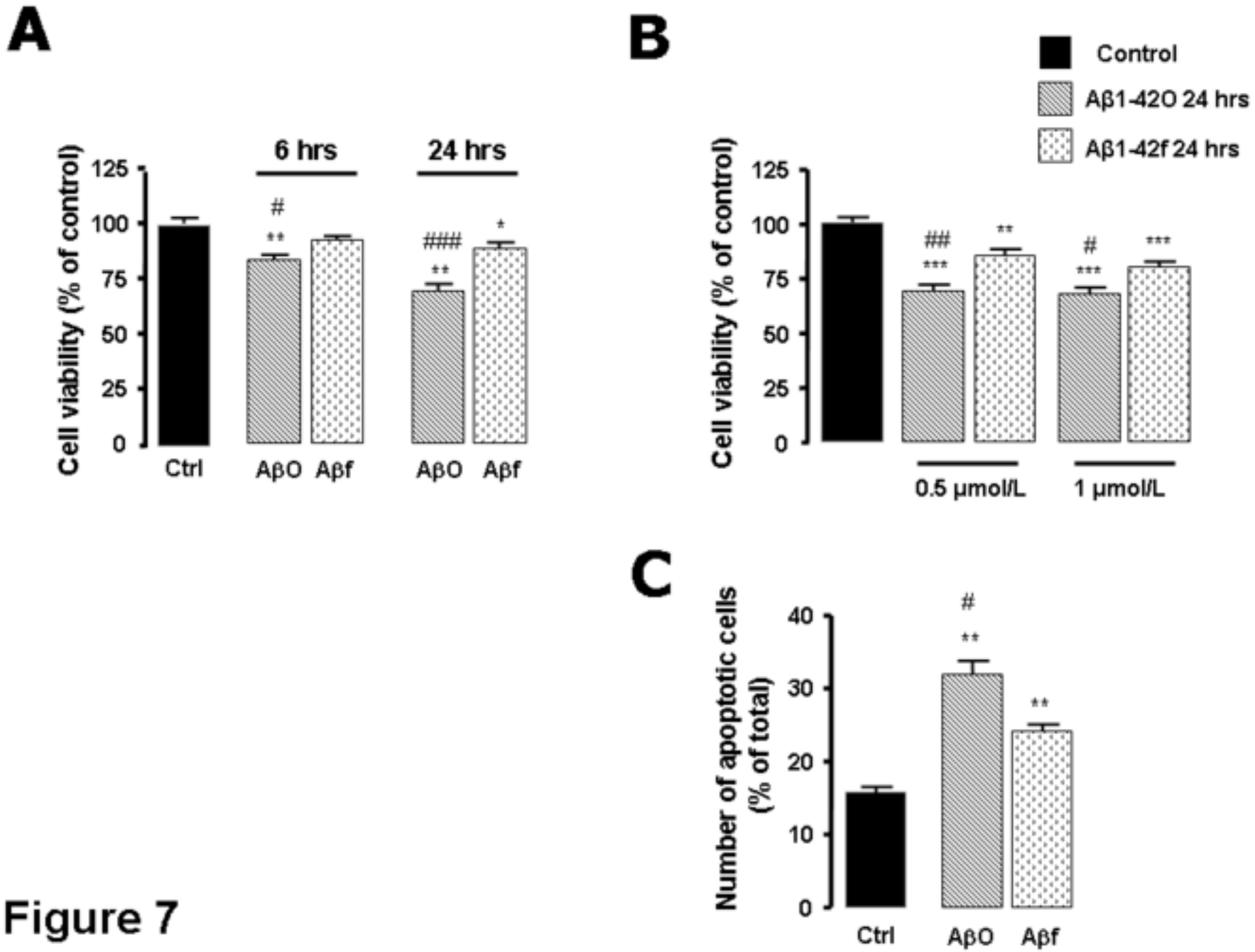


Figure 7

Figure
Click here to download high resolution image

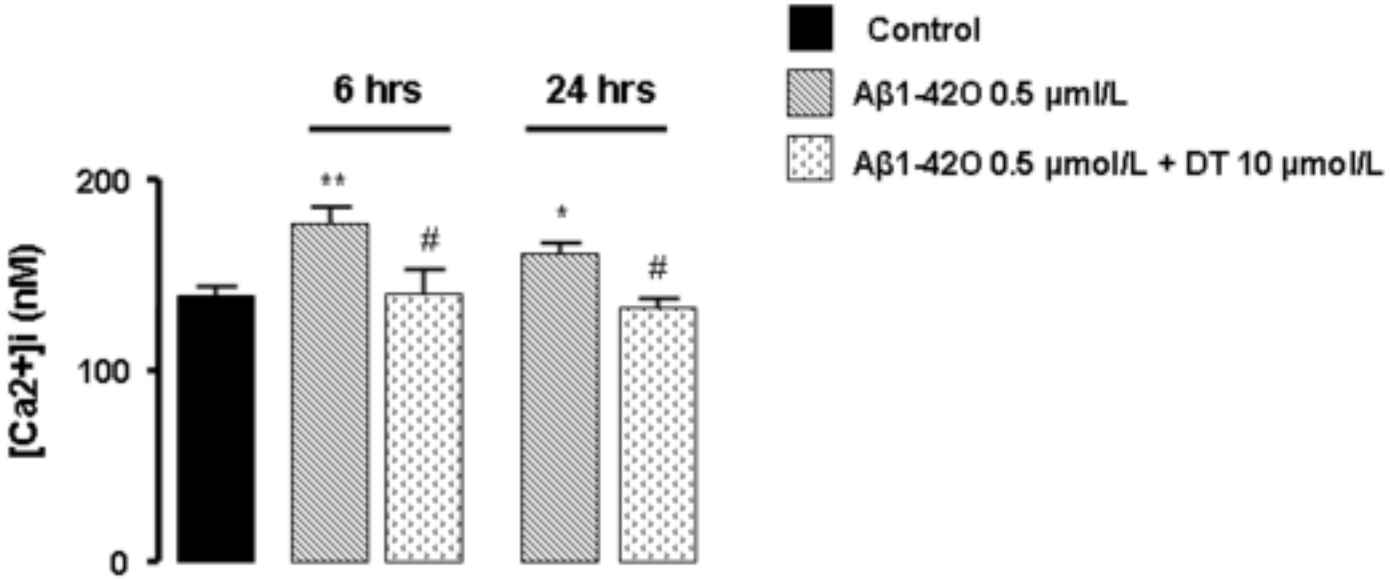


Figure 8

Figure
Click here to download high resolution image

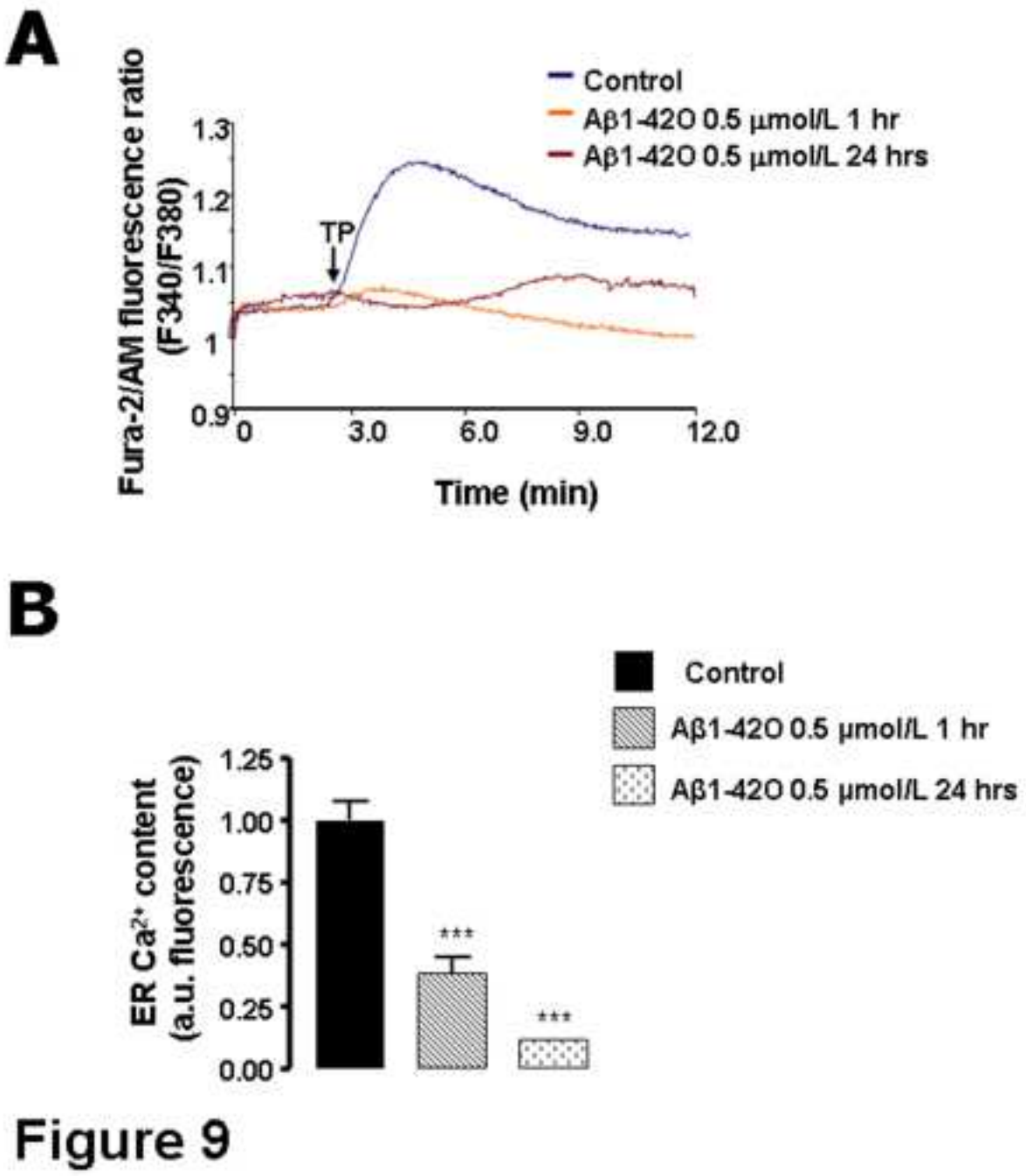


Figure 9

Figure
Click here to download high resolution image

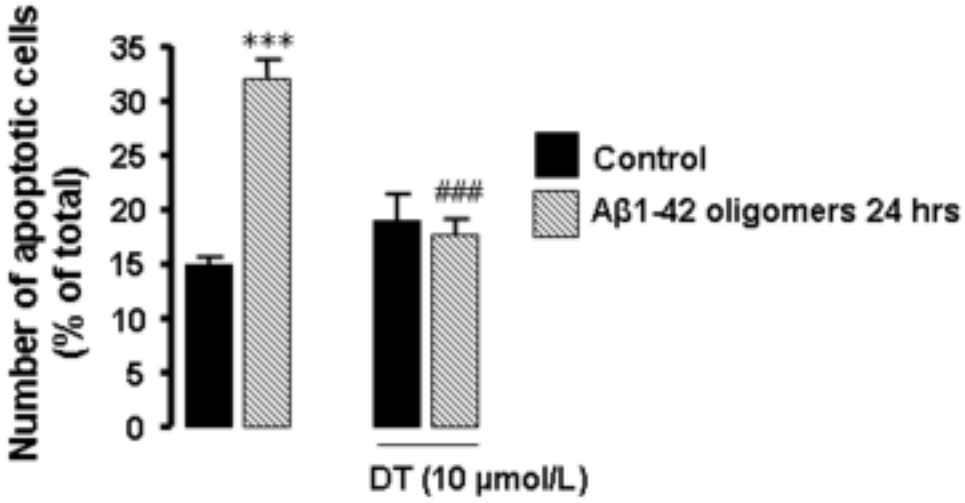


Figure 10

Figure
Click here to download high resolution image

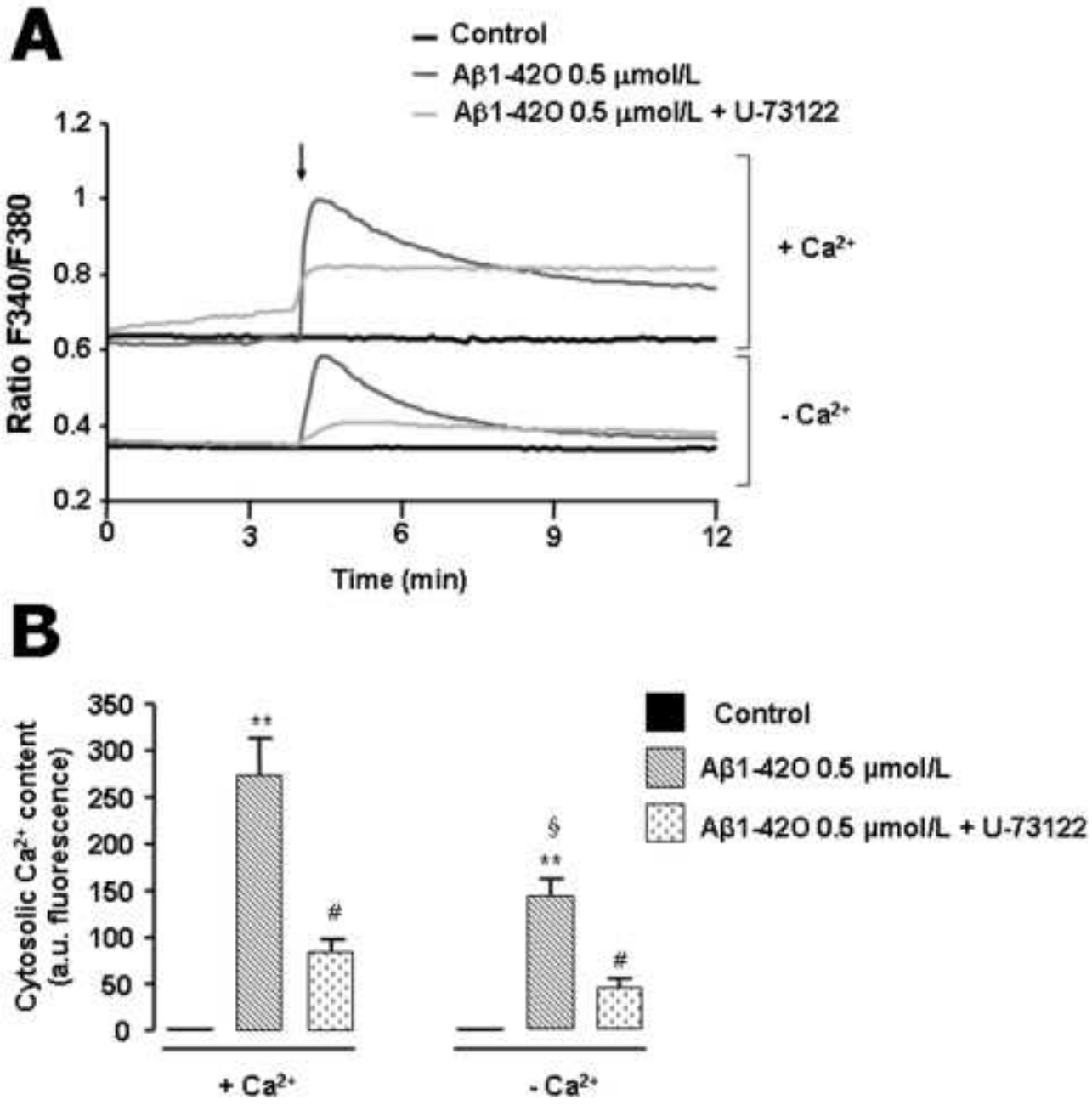


Figure 11

Figure
Click here to download high resolution image

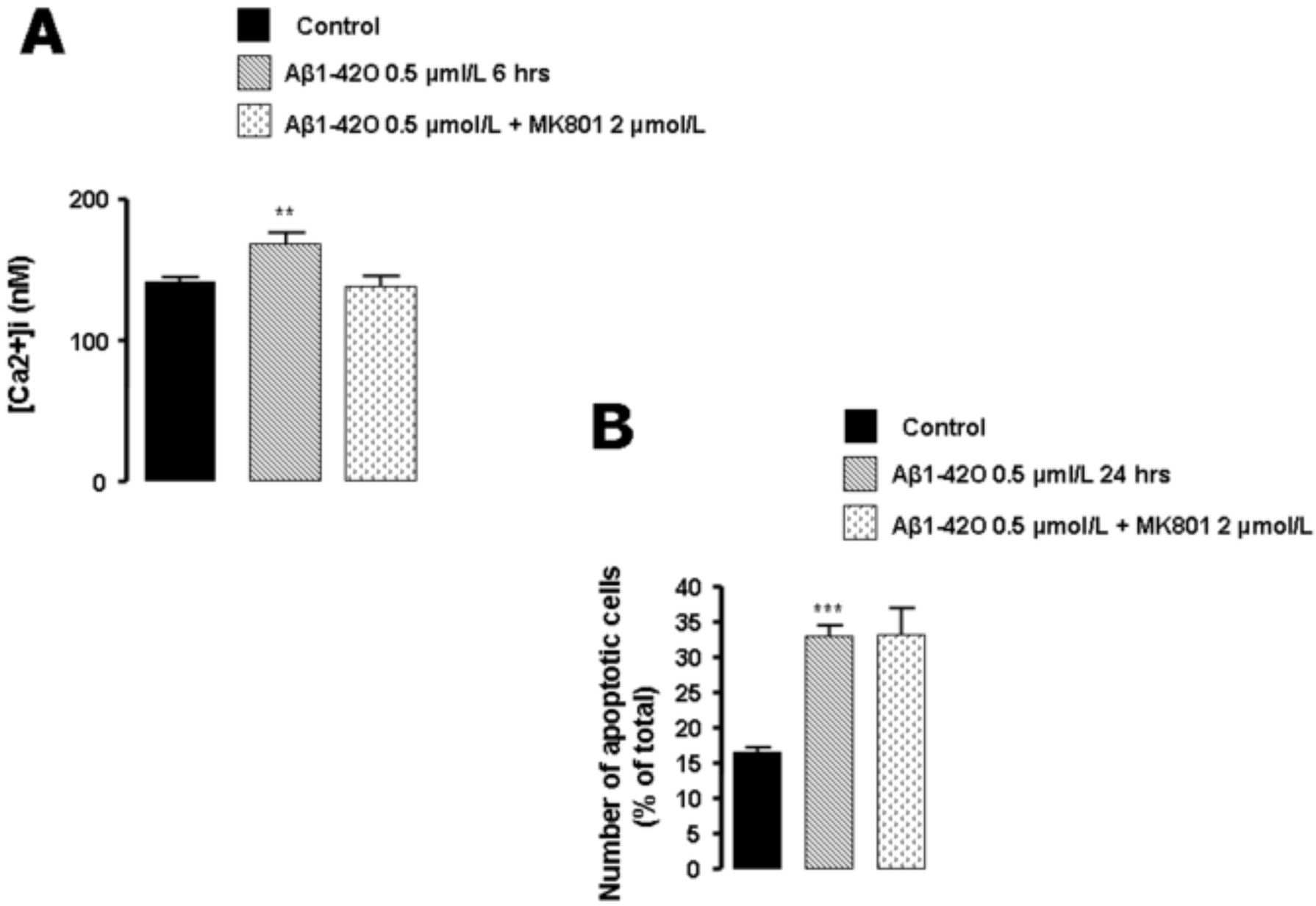


Figure 12